Use of Hematological Parameters to Predict Disease Resilience of Pigs in Commercial Environments

by

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ABSTRACT

High health status in nucleus and multiplication farms at the top of swine breeding pyramid is a barrier to genetic selection of disease resilient pigs. This resulted in a gap between the performance measured in the breeding company and the performance observed at the commercial level. At this time practical tools to select for resilient pigs do not exist. The overall objective of the thesis was to identify key differences in the complete blood count (CBC), namely blood erythrocyte, leukocyte and platelet concentrations between resilient and susceptible animals before and after natural challenge and to assess the potential of complete blood count (CBC) as a tool for breeders to select disease resilient animals in a high health environment.

We studied 893 high health crossbred (Landrace x Yorkshire) barrows from multiplication farms. These barrows were introduced in batches and exposed naturally to multiple diseases simultaneously in a test station. Natural disease challenge was established using seeder pigs to simulate high disease pressure typical of a commercial situation. Performance traits (growth rate and treatment rate) were assessed and used to classify pigs into resilient and susceptible groups. Profiling of 29 haematological parameters was performed on whole blood samples collected from individual pigs before and after pathogen challenge in the natural challenge model.

In chapter 3, in contrast to resilient pigs, susceptible pigs were found to have a low neutrophil concentration before challenge and a persistently higher concentration of neutrophils after disease challenge. This result was supported by the persistently higher expression of inflammatory cytokine genes such as *TNF-α* (P=0.08)*, IL-8* and *IL-1B* (P=0.09) in susceptible pigs after disease challenge. Although differences in red blood cells, platelets and their related traits before and after challenge did not provide any significant insights for the resilience trait, hemoglobin (FDR=0.05), MCV (FDR=0.02), together with WBC (lymphocytes (FDR=0.04) and monocytes (FDR=0.04)) were found to be significantly different between groups before challenge.

These CBC traits were therefore potential predictors of resilience at the commercial level in high health farms. This result led to the study in chapter 4, where principal component analysis was used to reduce the dimensions of the CBC data before challenge. A linear prediction model was then trained using the generated principal components and stepwise feature selection. In this study, different resilient/susceptible classification methods were used and resulted in different proportions of animals in these two groups. Among these methods and their respective prediction models, method B yielded an average 55.0% prediction accuracy that was significantly higher ($P<0.004$) than prediction accuracy of random classifier, 50.0%. Although the accuracy is relatively poor the predicted resilient group in method B showed significantly lower treatment rate ($P=0.05$) and shorter days to market ($P<0.05$) that looked promising in terms of its practical use in the field.

The findings from this thesis provide evidence that pre-challenge CBC could potentially be used by breeders in the classification of resilient and susceptible pigs in genetic nucleus and multiplication farms with high health status although further investigation is warranted to validate prediction results. Resilient and susceptible pigs showed different CBC and cytokine gene expression profiles before and after disease challenge. These differences may explain the disease/infection outcome of these two groups of pigs and could be developed as predictors of resilience.

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ADG	average daily gain
AGP	alpha(1)-Acid glycoprotein
AIC	Akaike Information Criterion
AMIR	antibody-mediated immune response
APC	antigen-presenting cell
APP	*Actinobacillus pleuropneumoniae*
B1	Blood 1
B3	Blood 3
B4	Blood 4
CBC	complete blood count
CBV	Cross-bred Breeding Value
CDPQ	Centre de developpement du porc du Quebec inc.
CHCM	Cell hemoglobin concentration mean
CMIR	cell-mediated immune response
CRP	C-reactive protein
CRSAD	Centre for Research in Animal Science Deschambault
EBV	Estimated Breeding Value
ECF18	*Escherichia coli* F18
FCR	feed conversion ratio
FEC	fecal egg count
GN	genetic nucleus
GWAS	genome-wide association studies
Hb	hemoglobin
HCT	hematocrit
HDW	hemoglobin distribution width
HEWL	Hen Egg White Lysozyme
IFN-y	interferon-gamma
Ig	immunoglobulin
IL-1B	interleukin-1B
LDA	linear discriminant analysis

LUC	large unstained cells
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MHC	major histocompatibility complex
Mhyo	*Mycoplasma hyopneumoniae*
miRNAs	micro RNA (ribonucleic acid)
MPV	mean platelet volume
mRNA	messenger RNA (ribonucleic acid)
NDCM	natural disease challenge model
NK	natural killer cell
NLR	neutrophil-lymphocyte ratio
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBML	peripheral blood mononuclear leucocyte
PCA	principal component analysis
PCT	plateletocrit
PCV	packed cell volume
PCV2	porcine circovirus type 2
PCVAD	porcine circovirus associated diseases
PDW	platelet distribution width
PED	porcine epidemic diarrhea
PMWS	post-weaning multi-systemic syndrome
PRDC	porcine respiratory disease complex
PRR	pattern recognition receptors
PRRS	porcine reproductive and respiratory syndrome
qPCR	Quantitative polymerase chain reaction
RBC	red blood cells
RDW	red blood cell distribution width
RES	resilient
RMSE	root mean squared error

SNPs	single nucleotide polymorphisms
SPF	specific pathogen-free
SSC4	*Sus scrofa* chromosome 4
SUS	susceptible
TGE	transmissible gastroenteritis
TH1	Type 1 T helper
TH2	Type 2 T helper
TNF	tumor necrosis factor
WBC	white blood cells

Chapter 1. Application of Complete Blood Count as a Measure of Immune Responsiveness for Prediction of Disease Resilient Pigs in a Natural Challenge Model

1.1 Introduction

The swine industry contributes substantially to the Canadian economy, in 2015 generating revenue of $3.43 billion in pork exports. The pork sector accounts for 30% of total livestock shipments and was the 4th largest agricultural industry in Canada, after canola, dairy products and cattle. Swine producers are primarily located in the Quebec-Atlantic provinces (37.9%), Saskatchewan-Manitoba provinces (27.4%), Ontario (21.0%), followed by British Columbia-Alberta (13.7%) (Statistics Canada, 2015; Canada Pork International, 2015).

From 1921 to 2011, the number of pig farms in Canada went from 8.1 per 100 inhabitants to 0.6 per 100 inhabitants, which represents a trend of consolidation and vertical integration in the industry (Statistics Canada, 2015). As pig farms have grown larger over the past few decades, large pig populations concentrated in a few areas pose a real challenge in managing swine health and farm biosecurity. Conventionally, swine health is maintained through several health strategies. The control of endemic diseases is usually done by maximizing immunity and minimizing the challenge or by correcting environmental-management deficiencies that will restore the herd health (Friendship, 2017). Alternatively, there are pathogens that can be eliminated through the use of medication, or depopulation of infected herds and repopulation with new animals free from the disease (e.g. transmissible gastroenteritis and porcine reproductive and respiratory syndrome (PRRS)), or exposing the herd on purpose to get herd immunity. The third strategy is to prevent the introduction of pathogens into the herd by applying key biosecurity measures, such as careful

management of replacement stock, quarantine procedures, prevention of disease transfers from trucks and fomites etc. (Friendship, 2017).

Vaccination is also a key component in maintaining and increasing herd immunity. Decisions on vaccination have to be assessed on a herd basis and therefore disease status in the farm has to be updated frequently. The decision is also made after consideration of the cost, efficacy and return on investment of the vaccine and also the risk of reoccurrence of the disease (Friendship, 2017). Medication, such as use of antibiotics, has been used strategically in feed, water and by injection to help reduce the negative economic impact of diseases and also to relieve the suffering of the animals.

The swine industry in North America has been plagued by numerous endemic and epidemic diseases that cause swine producers and the industry a substantial financial loss as well as welfare issues. An emerging disease, porcine epidemic diarrhea (PED) spread rapidly through the United States, causing high mortality and morbidity in the US pig production, and subsequently spread to Canada and Mexico in 2014 (Lee, 2015). More than 8 million newborn piglets died in the US in 2013 as a result of PED (Lee, 2015). Canada had its first case in January 2014, in a 500-sow farrow-to-finish herd in southwestern Ontario (Ojkic et al., 2015). PED is now globally recognized as an emerging and re-emerging disease and it has become a major financial issue for the swine industry worldwide (Lee, 2015). On the other hand, PRRS is a major endemic disease caused by a primary infection by PRRS virus (PRRSV), which has a major economic impact. For example, productivity losses for the US swine industry have been estimated to be $664 million annually (Holtkamp et al., 2013). The virus causes reproductive failure, respiratory disease, and growth retardation in the pigs. In addition to direct production losses, PRRSV significantly increases animal health costs for pharmaceuticals, biologicals, and diagnostics as well (Holtkamp et al.,

2013). However, due to the high degree of genetic variation in field PRRSV, current PRRS modified live vaccine (MLV) and killed vaccine confer only limited protection and fail to prevent the propagation of the infection (Thanawongnuwech and Suradhat, 2010; Charerntantanakul, 2012; Li et al., 2014). Moreover, like many other MLVs, PRRSV-MLVs have safety concerns including vertical and horizontal transmission of the vaccine virus and several documented incidences of reversion to virulence (Renukaradhya et al., 2015). Swine health status is further exacerbated by the banning of antimicrobial growth promoters (AGP) in Europe in 2006 which is due to the pressure to reduce antibiotic use in pig production worldwide (Cogliani et al., 2011). Occurrence of these diseases is a constant reminder to the swine industry of the importance of swine health and biosecurity in pig production, and more importantly novel approaches to solve the problem have to be encouraged.

As resistance traits are difficult or impossible to measure directly, immune responsiveness has thus been proposed as an indirect indicator of disease resistance in animals (Biozzi et al., 1980; Gavora and Spencer, 1983; Rothschild, 1989). Immune response measured from various immune traits that define the immune capacity of an animal determine the infection/disease outcome of an animal. The potential application of using these immune traits in the breeding scheme to select for healthier pigs that are less susceptible to infectious agents is an emerging trend in pig breeding (Flori et al., 2011). The selection of these immune traits is not easily achieved with the highly intricate and interactive nature of these traits. Ultimately, the choice of immune traits to be included in the breeding scheme will be based primarily on our knowledge of the immune system in pigs.

Classically, the immune system can be divided into innate and adaptive immunity. Innate immunity is delivered by cells such as phagocytes, natural killer cells, dendritic cells and

circulating proteins such as the complement system and several antimicrobial peptides (Abbas and Janeway, 2000). Innate immunity is an essential feature of the immune system where it is the front line of host defense after physical barriers such as skin break down. However, innate immunity does not guarantee a complete elimination of these microorganisms as there are some which are not recognizable.

Therefore, lymphocytes of the adaptive arm of the immune system provide extra protection especially against the reinfection by the same microorganism. Adaptive immunity can further be divided into antibody-mediated immune response (AMIR) and cell-mediated immune response (CMIR). AMIR protects the host against extracellular pathogens (typically bacteria), whilst CMIR protects the host against intracellular pathogens (e.g. viruses and intracellular bacteria and parasites). Even with these defense systems, several swine viral pathogens have been known to evade and replicate within the host by modulating the immunity of susceptible pigs. These viruses modulate innate and adaptive immunity by altering the cytokine patterns of macrophages and dendritic cells, as well as by modifying expression of molecules in antigen presentation that assist propagation of these viruses in the host. Examples of these viruses include PRRSV and porcine circovirus type 2 (PCV2). These viruses are able to cause immunosuppression by inducing strong regulatory cytokine response, via production of IL-10 in an infection (Mateu and Diaz, 2008; Darwich and Mateu, 2012).

This review will first focus on explaining the differences of the terms: resistance, susceptibility and resilience with some examples for each term. Then, we will look into the different types of conventional challenge models, challenges in their setup and weaknesses of each model, and how a new approach to resilience and a natural disease challenge model can help in exploring the resilience trait. We will also be discussing immune responses to major swine diseases

and the utilization of these responses as biomarkers for resistance/resilience, and the potential use of complete blood count parameters as resilience biomarkers.

1.2 Disease Resilience Trait

1.2.1 Definitions: resistance, susceptibility and resilience

Resistance has been defined as the ability of an animal to suppress establishment and development of an infection (Albers et al., 1984). Alternatively, it could be a situation where target tissue or cells of a resistant animal does not possess a pathogen receptor that would enable binding and entry of a pathogen into the cells as the first step of an infection (Plastow, 2016). This can also be referred to as true resistance where animals are completely unaffected by exposure to a pathogen. A classic example of a true resistance in pigs is *Escherichia coli* F18 (ECF18) associated diarrhea (Meijerink et al., 2000). Piglets that are resistant to ECF18 do not express a specific gene: fucosyltransferase 1 (*FUT1*) that relates to a specific receptor on the gut epithelium. As a result, they lack the adhesion site for the fimbriated ECF18 on the gut epithelium post-weaning. Genetic selection of ECF18 resistant pigs successfully reduces mortality which could be more than 20% in a naïve herd and modulates a potential loss in growth rate in pigs that survive (van der Steen et al., 2005).

On the other hand, susceptibility describes a direct opposite of the definition of resistance. Due to inability to reject the entry of a pathogen or to suppress development of an infection, a susceptible individual thus may succumb to the infection or if it survives, experience a reduction in growth rate and also in body weight over time compared with a resistant individual. Host genetic variation for susceptibility has been observed in many diseases (Bishop, 2010). For example, variation in susceptibility towards tuberculosis in cattle can be observed when infected cattle

experienced different impact of the disease and recovery time, despite control of various aspects such as the management, pathogen burden and environment (Allen et al., 2010). In pigs, PRRS challenge experiments conducted at Kansas State University also revealed that despite a well-controlled environment, viral load and growth rate in the study pigs differs greatly (Boddicker et al., 2012).

This variation in susceptibility is often polygenic in nature (Plastow, 2016). A polygenic trait is a trait that is controlled by large number of genes usually distributed across the genome where each gene explains a small proportion of the variation of the trait (Plastow, 2016). By using genome-wide association studies (GWAS), researchers have been able to use sequence variations (mainly single nucleotide polymorphisms, SNPs) in the whole genome, together with the phenotype and pedigree information, to perform association analysis and to identify genes that are important for the traits of interest in pigs such as boar taint, fertility, body composition and structural soundness etc. (Grindflek et al., 2011; Fan et al., 2011). In swine diseases, a region on *Sus scrofa* chromosome 4 (SSC4) was identified to have explained more than 10% of the variation in cumulative PRRS virus load post-infection (Boddicker et al., 2012). This variation can also be termed as oligogenic as this genomic region has been found to explain a relatively large amount of the variation (Plastow, 2016). The family of genes in the same region has also been associated with a higher weight gain and innate immunity of grower pigs (Rowland et al., 2012). This result suggests that it is possible to select for a commercial pig which is less susceptible to PRRS viral infection. In a separate study, a significant genomic component associated with PRRSV antibody response and number of stillborn was detected in data collected from sows in farms undergoing a PRRS outbreak (Serao et al., 2014). In these sows, 40% of the genetic variation in PRRSV antibody

response can be explained by two regions on SSC7 indicating the potential of antibody response to be used as an indicator of the impact of PRRS on reproductive traits (Serao et al., 2014).

There is a tendency within the industry to think of a resistant animal which is completely unaffected by a pathogen and indeed there are some examples of disease resistance in livestock, e.g. ECF18 (above). However, owing to the polygenic nature of the trait, breeding an animal with resistance to most diseases may not be achievable. Instead, an alternative may be to improve 'generalized immunity' of an animal where there is an increased ability of animals to respond to multiple pathogens and reduction of the impact of subclinical infections (Bishop, 2004). This is now termed resilience, "an ability of animals to respond to any infection in a way that minimizes the impact of disease" (Plastow, 2016). The impact of disease, or the animal productivity has been the centre of the definition of resilience. Productivity can vary from slaughter weight, days to market, average daily gain (ADG) and feed conversion rate (FCR) for a finisher pig; or it could be litter size, weaning weight, and percentage of stillborn for a sow in a commercial farm. Thus, instead of focusing only on the cost of treatment and mortality as in resistance, resilience takes into consideration the loss of potential growth or the economic impact of morbidity in the event of a disease occurrence (Plastow, 2016).

Furthermore, pathogen burden is not required in the measurement of resilience. By using a simulation, resilience is determined to be useful and practical in the selection of resistance without the need for records of pathogen burden, although these records if available, can further increase the accuracy of selection (Mulder and Rashidi, 2017). Often, the collection of phenotypes such as pathogen burden is a costly and difficult procedure. Using field samples, there are problems such as incomplete exposure to infection, low precision in diagnostic tests and variation in disease challenge that tend to reduce heritability of the resistance traits (Bishop and Woolliams, 2014).

Resilience is thus deemed to be a more realistic way of studying the response of animals towards an infection.

There have been numerous research studies of the resilience trait in different species in the past, yet the results have been mixed. In cattle, a test that measures both AMIR and CMIR has been created and used in cattle in identifying individuals with high or low immune response (Mallard et al., 1992; Sarker et al., 2000; Heriazon et al., 2011). By using this test, Canadian Holstein cows that have high AMIR and CMIR are found to have a lower occurrence of mastitis, improved vaccine response and increased milk and colostrum quality (Wagter et al., 2000; Thompson-Crispi and Mallard, 2012). Early work of resilience in pigs by the same group also focussed on the measurement of immune response, namely innate and adaptive responses. Pigs were selected based on their level of immune response to Hen Egg White Lysozyme (HEWL) challenge. However, after several generations of selection for high immune response to HEWL, these pigs were found to have a higher incidence of arthritis after *Mycoplasma hyorhinis* challenge (Wilkie and Mallard, 1999). Henryon et al. (2006) found no genetic associations between baseline levels of a number of immune traits and resistance to respiratory, lameness and other diseases. On the other hand, other researchers reported a negative genetic association between some immune traits and weight gain of pigs in a lower health status associated with commercial farms, indicating that genetic variability for this trait may be better expressed in low health environments (Clapperton et al., 2009). A wide range of immune parameters have been shown to be moderately to highly heritable (Flori et al. 2011, Henryon et al. 2006, Clapperton et al. 2008). So far, these mixed results have not led to the creation of a practical tool and there is no consensus among scientists about which specific immunity traits to use in pig breeding programs.

1.3 Disease challenge models

In the pig production pyramid, genetic improvement is normally done in a clean environment where this high health status is maintained with strict biosecurity, vaccination and management procedures. Decisions in genetic selection are usually made based on the performance potential measured under high health conditions at the top of the production pyramid. As the health status degrades going down the production pyramid, a gap exists between the performance stated in the breeding company and the performance observed in the commercial level (Plastow, 2016). The high health status in the genetic nucleus and multiplication farms at the top of the swine production pyramid is a barrier to phenotypic selection of disease resilient pigs that perform better in the lower health commercial level. However, whilst there are absolute advantages in maintaining high biosecurity within the farm as it favours genetic and phenotypic selection of certain performance traits such as feed conversion rate (FCR), average daily gain (ADG), reproduction traits, carcass traits etc., it poses an obvious weakness in identification and selection of animals that are disease resilient.

In the studies related to resistance traits in animals, researchers have been relying on a few disease challenge models. In the first type of model, data are harvested from farms with endemic diseases, such as mastitis and nematode infections; or epidemic diseases, such as bovine tuberculosis outbreaks (Bermingham et al., 2013). The collection of field samples is often 'opportunistic' in nature (Bishop and Woolliams, 2014). Thus, data collected from such an experimental design tends to be costly and difficult logistically (Bishop and Woolliams et al., 2014). Chances of misclassification of animals could be high with a low sensitivity diagnostic test, or when an exposure to infection is unknown. In both cases, estimated SNP effects will be reduced and biased (Bishop and Woolliams, 2014). A second model uses a disease-specific approach where

animals are challenged artificially using a standardized dose of a specific pathogen administered intra-nasally, intramuscularly or by ingestion to create an infection or disease condition. This type of challenge model is often smaller in scale, and the number of pathogens that can be tested are normally restricted to only one or sometimes two. In the third model, a large scale experimental design where animals are subjected to standardized environment and artificial disease challenge that is deliberately created and which it has been proposed as an ideal model for developing genomic predictors of resistance (Bishop and Wooliams, 2014). Compared to the second model, the third model is relatively larger in scale and an increased level of difficulty in setting up the model and higher cost involved. This type of challenge model may be more feasible in fish (Wetten et al., 2007; Moen et al., 2009) and chickens (Pinard-van der Laan et al., 2009), it is comparatively harder to emulate in terrestrial mammals (Bishop and Woolliams, 2014). It is thus rare in pigs as it requires large-scale funding and collaborations between several partners, seen in the study of PRRS disease in swine (Boddicker et al., 2012).

A natural disease challenge model (NDCM) has therefore been created with the aim to overcome some of the weaknesses of conventional challenge models and to explore disease resilience in pigs. This is the model that my thesis work is based on. A road map proposed for the application of animal genomics for animal health suggested that it could be more productive to study many diseases within the same population than to oversee many smaller disease-specific studies (Archibald et al., 2008). Furthermore, it is also important to recognize the co-infection of multiple pathogens in commercial production and its combined detrimental effect in pigs. This is demonstrated in the porcine respiratory disease complex (PRDC) where *Mycoplasma hyopneumoniae* potentiates the effect of PCV2 and PRRSV infections (Chae, 2016). Another example would be the porcine circovirus associated diseases (PCVAD), which are the combination

of different diseases attributed to porcine circovirus. Concurrent viral or bacterial infections such as PRRSV, *Mycoplasma hyopneumoniae, Haemophilus parasuis* and *Streptococcus suis* often accelerate the onset, increase the severity and prolong the duration of PCVAD (Opriessnig and Halbur, 2012). Recognizing these insufficiencies in conventional challenge models, NDCM uses the concept of mixed infections by introducing different swine diseases using seeder pigs from different commercial farms (Plastow, 2016). Thus, by using NDCM, genetic improvement of 'generalized immunity' in animals in response to various types of pathogens in the low health commercial environment can be explored (Bishop and Woolliams, 2004).

Furthermore, the challenges of collecting data from the field and the issue of an underestimated heritability of relevant traits due to a biased estimated SNP effect can be overcome by creating an environment where pigs are subjected to a more standardized challenge environment in NDCM. As mentioned, this type of experimental design requires large-scale funding and partnership between industry and academia, NDCM is currently used in studying resilience in pigs in Canada (Dyck et al., 2016; Plastow, 2016). More details on NDCM can be found in Chapter 2 of this thesis.

1.4 The immune system

1.4.1 Immune system overview

Skin and all mucous membrane in the body openings form external barriers and provide a first line defence against pathogens. These mechanical barriers are assisted by chemical substances such as acid, mucus, enzymes and hair-like structures, e.g. cilia in the bronchi, to prevent pathogens from entering the host systems.

Upon breakdown or evasion by pathogens of this first line of defence, the body triggers an innate response that recruits greater numbers of white blood cells such as monocytes and neutrophils from the blood circulation that are actively phagocytic (Figure 1.1). Also, production of chemical mediators such as the complement system, a series of more than 30 proteins that react with one another to damage bacteria by creating pores in the bacterial membrane; specific immunoglobulins (Ig) which coat bacteria and render them inactive or more vulnerable to phagocytes, in which both processes are called opsonization. This inflammatory response also includes the production of histamine by mast cells, which promotes vasodilation and a diverse host of chemical agents designated as cytokines (e.g., interleukins, interferons), which attract immune system cells, promote fever and inhibit bacterial and viral replication. All these changes form the second line of defense against many common bacteria. Cells involved in innate immunity use pattern recognition receptors (PRR) to identify the pathogen-associated molecular patterns (PAMP) present on the microbes (Abbas and Janeway, 2000). This is essential especially in the recognition and elimination of extracellular foreign microorganisms. Binding of the bacteria to the receptor triggers the phagocytic process and the release of cytokines, key regulators of immune responses. Cytokines are potent immunomodulatory molecules that act as mediators of inflammation and the immune response. Innate response is relatively fast (within 4 hours) as its reaction does not depend on previous exposure to the pathogens. Closely integrated with adaptive immunity, innate immunity initiates and determines the subsequent direction of the adaptive response.

As the innate defense system may not be able to eliminate infectious organisms that are not recognizable or that are able to evade host innate immunity, the host then mobilizes a defense mechanism with higher specificity, this is the adaptive immune response which consists mainly of

T lymphocytes and B lymphocytes, which is also considered the final line of the defense system (Figure 1.1).

The CMIR of adaptive immunity is characterized by T lymphocytes. After completing their development in the thymus, T-cells enter the bloodstream and are induced by the peptide:MHC complex on the surface of APC to proliferate and differentiate into effector T-cells. These effector cells include cytotoxic T-cells, killer cells that destroy foreign antigens or cells containing foreign proteins upon recognition; helper T-cells produce cytokines that promote the proliferation of both cytotoxic T-cells and B-cells; and suppressor T-cells that produce inhibitory cytokines that stop the proliferation when the invading pathogens have been destroyed. CMIR responds effectively towards intracellular pathogens and is characterized by the production of IL-2, interferon-gamma (IFN-y) and antibodies of IgG2 isotype (Estes and Brown, 2002; Zhu and Paul, 2008).

In AMIR, the second class of adaptive immunity, B lymphocytes produce antibodies, proteins that are capable of combining with and inactivating foreign antigens. Binding of antibody inactivates viruses and microbial toxins by disrupting their ability to bind to receptors of host cells. Antibody binding also marks the invading pathogens which in turn enhances the phagocytic ability of cells involved in innate immunity. In the spleen, these B lymphocytes differentiate into plasma cells and memory B lymphocytes. Plasma cells are specialized to produce specific antibodies at a much higher rate than regular B lymphocytes. AMIR predominates in the host response towards extracellular pathogens and is characterized by the production of IL-4, IL-13 and antibodies of IgG1 isotype (Thompson-Crispi et al., 2012). Several other classes of immunoglobulins with antibody activity that are equally important in AMIR includes IgA, IgM and IgE. IgA is mainly distributed on mucosal surfaces and various types of secretion, such as saliva, colostrum, sweat and tears, whilst IgM and IgE are mainly distributed in the blood.

In adaptive immunity, the first encounter with the antigen that leads to the production of effector cells takes four to seven days after pathogen recognition. Contrary to innate response, both B and T cells produce memory cells that retain the capacity to recognize a specific foreign protein after the first encounter. This enables a more rapid production of effector cells with subsequent exposures to the same infectious antigen.

1.4.2 Immune responses as biomarkers of the resilience trait

Immune response to an infection or disease has long been proposed as an indirect indicator of disease resistance trait of an animal (Biozzi et al., 1980; Gavora and Spencer, 1983; Rothschild, 1989). Genetic variation in host susceptibility to infectious disease undoubtedly exists, largely contributed by the variation of host immune response to infection (Bishop, 2010). It is important to note that a higher level of immune responses may not translate to a better host defence as different types of immune response may be deployed to eliminate different kinds of pathogens (Roitt et al., 2001; Adamo, 2004). Moreover, excessive immune response might cause more harm than cure. This is evidenced in the example mentioned earlier, animals selected for higher immune response are more inclined to develop arthritis following *Mycoplasma hyorhinis* challenge (Wilkie and Mallard, 1999).

Several immune traits have been studied and linked to the occurrence and persistence of swine diseases. Lowe et al. (2005) has proposed a higher level of CMIR, as indicated by the secretion of interferon-gamma (IFN-y) secreting cells, decreases the reproductive clinical signs of PRRS in sows in commercial farms. In grower-finisher pigs, the variation of gene expression of all three cytokines, *IL-1B*, *IL-8* and *IFN-y* in serum has been significantly correlated with PRRS virus level and linked to the persistence of PRRS in pigs (Lunney et al., 2010). PCV-2 infected

pigs presenting with poor growth, interstitial pneumonia and sudden death were shown to exhibit strong response in the systemic concentrations of IL-10, IL-1B and IL-8 (Darwich and Mateu, 2012). An elevated level of C-reactive protein (CRP), an acute phase protein, and IL-10 have also been identified as an early sign of PCV2-infected piglets that subsequently developed severe post-weaning multi-systemic syndrome (PMWS) (Stevenson et al., 2006). Acute phase proteins are plasma proteins, produced by hepatocytes upon stimulation by pro-inflammatory cytokines, where the circulating concentrations are adaptively regulated in response to most forms of inflammation, infection and tissue injury. Links have also been established between some of these immune traits with the performance of pigs at the commercial level. Peripheral blood mononuclear leucocyte (PBML) subset, CD11R1+ cells consisting of natural killer (NK) cells and NK T cells and alpha(1)-Acid glycoprotein (AGP), another acute phase protein have also been suggested as predictors of pig performance in low health status in commercial farms (Clapperton et al., 2009). Here, we envision that resilience could be an outcome resulting from the combined effect of multiple immune biomarkers and cannot at present be accurately predicted based on evaluation of individual immune biomarkers.

In sheep, haematological parameters that include white blood cells have been researched to rank the resistance level to *Haemonchus contortus* in sheep in Australia (Andronicos et al., 2014). This is to complement the conventional selection strategy that involves differential packed cell volume (PCV) and fecal egg count (FEC) (Andronicos et al., 2014). In dairy cattle, a broad-based immune response, high immune response (HIR) technology has been developed that includes utilization of AMIR and CMIR in turn to develop a breeding index in the selection of Canadian Holsteins with reduced mastitis incidence (Mallard et al., 2011). This tool has also been

successfully marketed under the trade name IMMUNITY+® and implemented not only in the Canadian dairy cattle industry but worldwide (Semex, 2018).

1.4.3 Complete blood count

Complete blood count (CBC) has been an important extension of physical examination used by clinicians to monitor an immune response or state (Jones and Allison, 2007). It is used by clinicians to monitor disease progression, assess bone marrow and immunological functions, determine types of anemia etc. in both veterinary and human medicine.

In addition, immune response of animals can be evaluated using total white blood cells (WBC) and their differential cell concentrations, such as the neutrophils, lymphocytes, monocytes, eosinophils and basophils. The differential count by cell types is more important than the total WBC as an increase in a cell type and a decrease in another cell type can happen at the same time and not change the total WBC (Jones and Allison, 2007). Lymphocytes are predominantly B cells and T cells that play important roles in adaptive immune response.

Neutrophils migrate to the site of damaged tissue or inflammation within two hours of invasion by foreign material or pathogens (Jones and Allison, 2007). An elevated neutrophil count, or neutrophilia may occur in response to an inflammation, stressful conditions or during exercise (Jones and Allison, 2007). Pathological lymphocytosis is also found in cases of chronic viral infections, chronic pyogenic infections or autoimmune diseases (Jones and Allison, 2007). On the contrary, lymphopenia, the condition of having an abnormally low level of lymphocytes in the blood is the hallmark of viral infections with tropism for rapidly dividing cells, such as PRRSV and Porcine Parvovirus infections. PRRSV modulates host immunity by suppressing innate immunity causing an abnormality in B cell proliferation (Butler et al., 2014). Previous studies have

also reported the observation of an elevated *IL-10* mRNA expression in the thymus of PCV2-infected pigs that suffered from severe lymphopenia (Darwich et al., 2003; Kerarainen et al., 2008; Sipos et al., 2004). Monocyte concentration has also been reported to have a negative genetic correlation with pig performance under specific pathogen-free (SPF) environments (Clapperton et al., 2009).

Platelets have been discovered in recent years to play an important role not only in hemostasis but also in inflammation by influencing innate and adaptive immunities (Stoppelar et al., 2014). It has also been suggested that platelets are a biomarker for early sepsis recognition as they are involved in the sepsis pathogenesis and contribute to sepsis complications (Stoppelar et al., 2014). Platelets are also reported to have an active role in the activation of the complement cascade in vascular inflammation and thrombosis (Peerschke et al., 2010). Numerous inflammatory mediators secreted by platelets, that have no known function in haemostasis, modify leukocyte and endothelial responses to different inflammatory stimuli (Thomas and Storey, 2015). Mediated by platelet P-selectin, platelets form bridges between endothelium and leukocytes promoting inflammation (Thomas and Storey, 2015). PMWS-affected pigs are often found to be lymphopenic and thrombocytopenic with a relative and absolute monocytosis (Mendes et al., 2008; Sibila et al., 2004).

In animal science research, CBC measured early in life for selection of the residual feed intake of pigs could be effective as a few CBC traits were shown to have a substantial genetic component (Mpetile et al., 2015). As introduced above in sheep, besides using packed cell volume (PCV) and fecal egg count (FEC), a CBC profile that includes white blood cells, red blood cells and platelets have been used by researchers in developing a prediction algorithm to rank the

resistance of sheep to *Haemonchus contortus* and they are able to classify these sheep into resistant and susceptible groups with 80% and 100% accuracy respectively (Andronicos et al., 2014).

1.5 Conclusion

For many years, researchers have been focusing their efforts in finding the key towards resistance trait of a specific swine disease, however, breeding an animal with resistance to most diseases may not be achievable. Disease resilience therefore offers a pragmatic approach by focusing on the impact of performance from disease exposure or infection. This approach may be more straightforward to measure. Furthermore, these research studies may also have been constrained by the approaches used such as the conventional challenge model and the requirement to obtain pathogen burden within the animal. High cost and difficulty of obtaining such phenotypes limit the scale of the research and thus reduce the power of the research especially from a genetic point of view. NDCM thus presented an unprecedented platform for researchers to extract valuable phenotypes that includes the biological samples, performance and health records of over 3000 pigs.

1.6 Overall objectives and hypothesis

The overall objective of the thesis was to assess the potential of complete blood count (CBC) as a tool for breeders to select disease resilient animals in a high health environment, e.g. genetic nucleus and multiplication farms in order to close the difference in gap between the performance measured in the breeding company and the performance observed at the commercial level.

The specific objectives were:

1. To identify key differences in the CBC profile between resilient and susceptible animals before and after natural challenge (Chapter 3)
2. To determine whether the higher neutrophil-lymphocyte ratio (NLR) after natural challenge in susceptible animals is associated with the higher level of pro-inflammatory cytokine gene expression (Chapter 3)
3. To evaluate the potential of using CBC results to develop an algorithm to predict the resilience classification of pigs even before the natural challenge (Chapter 4)

As mentioned previously, immune traits have been associated with the occurrence and persistence of certain swine diseases (Lowe et al., 2015; Lunney et al., 2010; Darwich and Mateu, 2012; Clapperton et al., 2009). It could therefore be an indirect indicator to measure resilience trait in pig. CBC has long been used by clinicians to measure immune response in veterinary medicine. This test has been used by researchers to rank resistance level of *Haemonchus contortus* in sheep and to classify resistant and susceptible sheep with high accuracy (Andronicos et al., 2014). Based on the above findings, we postulated that resilient and susceptible pigs would express different immune response and that these different responses can be observed in the CBC. In addition, these differences in CBC may help identify and explain the ability of resilient pigs to recover from infection and reduce the impact of disease. Measurable differences in CBC before the occurrence of disease challenge can be used to develop a prediction algorithm for the resilience classification of the animals.

Therefore, the hypotheses of this work were: a) there are differences in CBC profiles between resilient and susceptible pigs before and/or after challenge; b) susceptible animals have higher levels of expression of genes for pro-inflammatory cytokines after natural challenge; c) pre-challenge CBC data can be used to predict the resilience classification of the animals.

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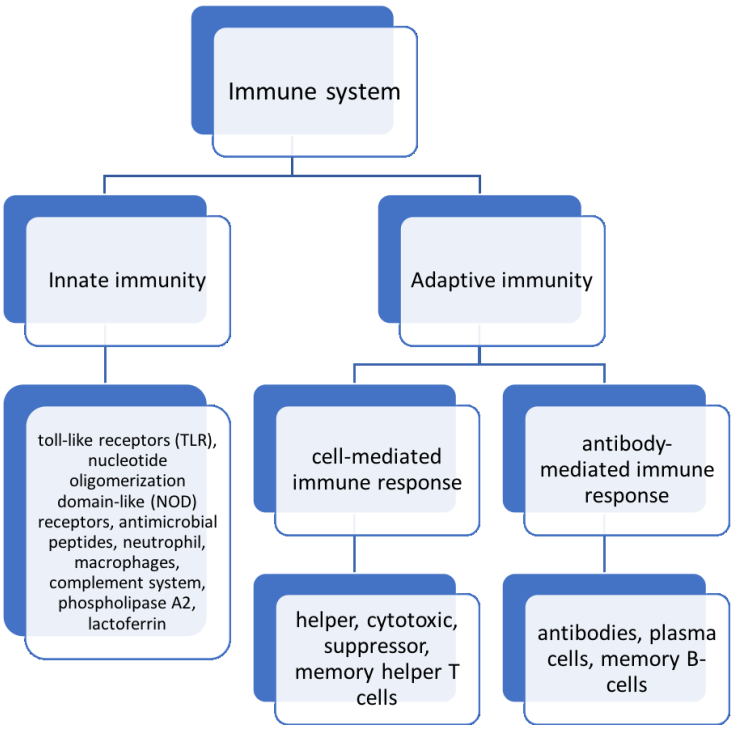
Figure 1.1 Components involved in innate and adaptive immunity



Chapter 2. Descriptive Analysis of Pig Performance, Mortality and Treatment Causes in Cycle One and Two of a Natural Disease Challenge Model

2.1 Introduction

The high health status in the genetic nucleus (GN) and multiplication farms at the top of the swine breeding pyramid is a barrier to phenotypic selection of disease resilient pigs that perform better in the lower health commercial level. These GN farms are normally located in a geographically isolated area, away from commercial farms, cities and highways, with the aim of minimizing any routes of transmission of pathogen/disease, as biosecurity is a major concern in these facilities. Disease prevention and control is the key to maintaining healthy genetic improvement in breeding farms.

However, whilst there are absolute advantages in maintaining high biosecurity within the farm as it favours genetic and phenotypic selection of certain performance traits such as feed conversion ratio (FCR), average daily gain (ADG), reproduction traits, carcass traits etc., it poses an obvious weakness in identification and selection of animals that are disease resilient. Under these high health conditions, disease resilience trait identification and selection would not be achievable. In the industry, breeders have been using semen of pureline GN herd sires to inseminate commercial sows at the commercial level. Performance traits of these crossbred progeny such as litter size, weaning weight, resilience, gilt health status and piglet survivability are then incorporated in the GN database. This has enabled breeders to derive a Cross-bred Breeding Value (CBV), together with pure bred Estimated Breeding Value (EBV). In this way the selection of the pure breed dam and sire in the GN farms has been enhanced to address the performance of the commercial pig (Evans, 2009). The latter is the overall measure of

improvement and is measured as the genetic merit of an animal, half of which will be passed on to its progeny. However, producers may be reluctant to use semen of pure GN herd sires in their breeding program as single sire mating may reduce litter size if the selected sire is subfertile. The potential economic loss in litter size may hamper the participation of producers and with the questionable sustainability of this method, breeders may not garner sufficient participation to derive a meaningful CBV. Alternatively, pedigree recording in the commercial level can still be implemented using mixed semen mating with the assistance of genotyping technology, however, this is not done because of the high cost incurred to genotype all the sires, dams and their offspring.

To help decipher the underlying mechanism of susceptibility or resistance to certain diseases, researchers used to collect performance data and biological samples from commercial farms undergoing a disease outbreak. As discussed in Chapter 1, pathogen exposure in the field environment and sensitivity of diagnostic tests tend to play crucial roles in determining the success in this type of challenge model. As indicated, heritability of disease resistance and the power of datasets under such conditions are often low (Bishop and Woolliams, 2010). Instead of relying on field data, researchers have also utilized a single-disease approach in an artificial challenge model where the pathogen is administered via injection, ingestion or nose-to-nose transmission. As discussed, this type of challenge model is often smaller in scale and not able to consider mixed infections or diseases with multi-factorial etiologies involving physical factors such as management, ventilation system, temperature, humidity, etc. as in the commercial situation.

In order to overcome these limitations of previous work, a natural disease challenge model (NDCM) was designed by researchers with the aim to explore disease resilience in pigs. Using a NDCM, naïve crossbred animals from high health multiplication farms were directly challenged with multiple swine pathogens introduced using seeder pigs from low health commercial farms in

a test station. Unlike an artificial challenge using a single pathogen in a clean research setting, NDCM recognized and harnessed the synergistic effect of a combination of pathogens that cause complex diseases such as porcine respiratory disease complex (PRDC), porcine reproductive and respiratory syndrome (PRRS) etc. as well as the environmental (barn & management) effects. Using this challenge model, both crossbred heterosis and pathogen synergy were considered.

The NDCM was designed to overcome the weaknesses of other challenge models or outbreaks and by exploiting the variation in performance of these multiplier pigs under low health conditions, valuable and real commercial phenotypes can be collected.

2.2 Materials and methods

Experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock (AUP00002227). Pigs were handled in accordance with the guidelines described by the Canadian Council on Animal Care (CCAC, 2009). The animal study was conducted at the Centre for Research in Animal Science Deschambault (CRSAD) quarantine and acclimatization unit and Centre de developpement du porc du Quebec inc. (CDPQ) test station in Deschambault (Quebec City, QC, Canada).

2.2.1 Natural Disease Challenge Model

A total of eight hundred and ninety three (n=893) high health nursery crossbred barrows of Yorkshire and Landrace lines from multiplier farms were enrolled in this study through PigGen Canada members. The multiplier herds were followed by a veterinarian for at least six months in order to confirm the absence or effective control of the following diseases: viral diseases such as porcine epidemic diarrhea (PED) (associated with porcine epidemic diarrhea virus (PEDV)),

transmissible gastroenteritis (TGE) (associated with transmissible gastroenteritis virus (TGEV)), porcine reproductive and respiratory syndrome (PRRS) (caused by porcine reproductive and respiratory syndrome virus (PRRSV)), porcine circovirus associated disease (PCVAD caused by porcine circovirus type 2 (PCV2)), swine influenza (caused by swine influenza virus); and bacterial diseases, such as atrophic rhinitis (caused by *Pasteurella multocida* with/without *Bordetella bronchiseptica*), Glasser's disease (caused by *Haemophilus parasuis*), swine dysentery (caused by *Brachyspira hyodysenteriae*), enzootic pneumoniae (caused by *Mycoplasma hyopneumoniae*) (Mhyo), severe pleuropneumonia caused by *Actinobacillus pleuropneumoniae* (APP), exudative epidermitis (caused by *Staphylococcus hyicus*); as well as ectoparasitic disease, such as sarcoptic mange (associated with *Sarcoptes scabiei* var *suis*).

These high health multiplier pigs were then introduced into the test station in batches of 60 or 75 pigs every three weeks. In total, fourteen batches of pigs were contributed by the seven different PigGen members and transported from their multiplier herds to the test station. The first seven batches (batch 1 – 7) were labelled as cycle 1 and the second seven batches (batch 8 – 14) were labelled as cycle 2. These pigs were weaned at multiplier farms at approximately 21 days of age and transported to CDPQ research farm in Deschambault, Quebec, Canada where the natural disease challenge model was established.

2.2.2 Natural disease challenge for cycle 1

After arrival at CDPQ in Deschambault, multiplier pigs in cycle 1 were placed in the nursery unit of the test station which functioned as a quarantine and acclimatization unit for approximately 19 days. Natural disease challenge began with the transfer of multiplier pigs from the nursery unit to the grower-finisher (GF) unit within the test station (Figure 2.1). In cycle 1,

four groups of seeder pigs with 67 pigs in total were introduced with the first four batches of test pigs. The first three groups included a total of 39 pigs. These pigs had low body weight with an average weight of 17.9 kg at 42 days of age upon entry, and they originated from commercial farms selected based on the history of diseases caused mainly by PRRSV in the farms. The last group of 28 seeder pigs were already in the finisher stage with an average body weight of 107.6 kg, selected from commercial farms with disease history of enzootic pneumonia caused by Mhyo. For batch 1- 3, multiplier and seeder pigs (first three groups) were housed in separate pens in the grower-finisher unit, these pens were arranged side by side to allow direct (nose to nose) pig contact. In batch 4, two seeder pigs from the fourth group were placed along with 7 – 8 multiplier pigs in the same pen to allow direct contact and to create cross infection. After establishing the disease status, the grower-finisher unit was run in continuous flow for disease retention and transmission. No seeder pigs were introduced after batch 4.

At the end of cycle 1, several changes were made to the experimental protocol to reduce both mortality and treatment rates in the test station. These changes included setting up a CRSAD quarantine and acclimatization unit in an independent building, halting introduction of seeder pigs from commercial farms, pulse feed medication during the grower-finisher phase and vaccination for PCV2 (Ingelvac CircoFLEX®) two weeks after entry.

2.2.3 Natural disease challenge for cycle 2

Learning from experience in cycle 1, a quarantine and acclimatization unit was setup in CRSAD, an independent building from the CDPQ test station (Figure 2.2). At entry, multiplier pigs were first placed in CRSAD for 3 weeks. During the second week in CRSAD, these pigs were vaccinated for PCV2. In this cycle, there was no introduction of seeder pigs in the CDPQ nursery,

instead each new batch of pigs was placed in a pen side by side with the old batch of pigs for a week, this arrangement of alternating pens between 2 batches of pigs facilitated cross infection (Figure 2.3). Cross infection between batches in the CDPQ nursery unit was repeated for every consecutive incoming batch of pigs. After 21 days in the nursery unit, the new batch of pigs was moved into the grower-finisher unit within the same test station building until slaughtering age or weight. Both nursery and grower-finisher units were run in continuous flow for disease retention and transmission.

2.2.4 Health status management

Exposure to targeted pathogens (PRRSV, influenza, Mhyo, APP) were checked by testing for antibodies to these pathogens using ELISA of serum collected from 16 to 20 pigs (4 pigs per pen) at 12 weeks post-arrival. Seeder pigs (in cycle 1) were bled within the first week after arrival to confirm the pathogens that were introduced into the test station.

Humane interventions were defined by the site Animal Protection Committee (APC) and an annual approval certificate with authorisation number 15PO283 was issued by Comité de Protection des Animaux (CPA). Reports of disease symptoms, treatments and mortality rates were prepared weekly by the veterinary advisory team and sent to the committee. Pigs identified as demonstrating disease or in ill health and exhibiting clinical signs were treated individually with injectable medication recommended and approved by the veterinarian in charge of herd health.

Water medication during the nursery stage and pulse medication in the grower-finisher feed were also administered to control disease morbidity and mortality in the test station. Welfare of the pigs, clinical signs and severity of diseases and response to treatment were among the main principles guiding the decision of euthanasia. The procedures for humane euthanasia were

described in a specific protocol assisted by digital images and videos. Pigs were observed for any distress at an unacceptable level and every pig that was unresponsive to treatment, i.e. did not recover or experience relief from pain, was euthanized. Several conditions warranted an immediate euthanasia without authorization of veterinarians: these included fracture or joint dislocation, non-weight bearing lameness that was non-responsive to treatment after 7 days, severe peripheral cyanosis accompanied with abdominal breathing etc.

Mortality in the subsequent analysis was defined as deaths that were due to infectious causes, this includes animals that were not thriving or were sick and were euthanized based on the humane endpoints specified in the approved protocol. Mortality that was due to non-infectious causes, such as traumatic injuries, herniation, sarcoptic mange and death during blood sampling were all filtered and excluded from the dataset.

2.2.5 Collection of performance and health data

Performance data was recorded for each individual pig. This included age and weight at entry (weaning) and every 3 weeks after entry until slaughter (in CRSAD, nursery and grower-finisher unit of CDPQ test station), slaughter age and weight, backfat and muscle thickness at slaughter, carcass weight and lean yield. Daily feed intake of pigs in the nursery unit was measured by pen and recorded manually. In the grower-finisher unit, the pig's individual feed intake was measured using an electronic device called Insentec IVOG system. This system recorded the daily amount and frequency of feed intake during each meal for each pig by detecting an electronic ID implanted in the ear of pigs. Pigs were marketed when they reached the targeted off-test live weight of 130 kg or after 24 weeks in the test station. Death age and weight of dead or euthanized pigs

were also recorded. Daily mortality and treatment rate and causes was recorded, compiled and assessed by the veterinary advisory team on a weekly basis.

2.2.6 Biological sample collection

Various biological samples were collected from the pigs, primarily blood samples using different types of blood tubes at different times during the pig production (Table 2.1). Owing to the differences in model designs of cycle 1 and 2, these samples were also collected at different places and/or times. After collection these samples were sent to University of Alberta (UofA), University of Saskatchewan (via UofA), Laval University, and University of Guelph for analysis of cytokines, complete blood count (CBC), blood transcriptome, genotype and natural antibodies as well as related studies of the immune system (subject of other work) (see Table 2.1).

2.2.7 Statistical analysis

Data for each measure of performance were described independently using PROC MEANS and PROC UNIVARIATE and presented as means±standard error of the means (SAS 9.1.3) (Table 2.2). The correlation between entry age and weight, slaughter age and weight, ADGs of nursery and grower-finisher phases were analyzed by Pearson Correlation Coefficients using PROC CORR (SAS 9.1.3) (Table 2.3). In addition, mortality and treatment records of pigs in both cycles were compiled and presented in Table 2.4.

2.3 Results and discussion

2.3.1 Performance variables across cycle 1 and 2

Generally, cycle 2 pigs had a higher entry weight of 6.69 kg compared to 6.31 kg for cycle 1. This difference of 380 g widened in consecutive weight records and ended up with a difference of 5 kg of pre-slaughter weight although pigs in both cycles were slaughtered at almost the same age, average of 182 days (Table 2.2).

Pearson correlation results indicated that entry weight had significant positive correlation with average daily gain (ADG) in nursery ($r=0.38$, $p\leq0.001$) and pre-slaughter weight ($r=0.21$, $p\leq0.001$) (Table 2.3). Bearing in mind that entry weight corresponds to weaning weight, significant correlation indicated that the higher the weaning weight, the higher daily gain in the nursery unit resulted in higher pre-slaughter weight. This finding was similar to the results of previous studies showing that pigs with higher weaning weight were able to reach slaughter weight earlier (Mahan and Lepine, 1991; Wolter and Ellis, 2001).

Entry weight, however, was not correlated to ADG during the grower-finisher phase ($r=0.06$, $p=0.22$), which meant that higher weaning weight may only be an advantage in the early stages for the pig (Table 2.3). It could probably be due to the high impact of disease challenge during nursery and grower-finisher phases that entry weight seemed to play a small role in daily gain of a later phase. It was also worth noting that influence of weaning weight on ADG could be diminished over time. Although having a value close to significance level ($p=0.05$), ADG in nursery was weakly correlated to ADG in grower-finisher ($r=0.09$), indicating a good growth rate in nursery phase did not guarantee a good growth rate in the grower-finisher phase. Again, disease challenge could be one of the contributing factors here as pigs that grow well in nursery phase may succumb to disease in the grower-phase phase and cause this weak correlation and vice versa. A

physiological phenomenon known as compensatory growth can also affect growth rate in the grower-finisher phase as a result of restriction of feed supply during the nursery phase (Heyer and Lebret, 2014). However, feed restriction in NDCM was not set up intentionally but was most probably a negative outcome due to an infection and/or disease. Other than disease factors, it was reported that growth rate in a later stage may partially be determined by the number of muscle fibers, which was associated with the genotype of pigs (Blunn et al., 1953; Dwyer et al., 1993). In other words, the genetic background of pigs may have influenced the ADG in grower-finisher phase.

Significant positive correlations were observed between ADGs during nursery ($r=0.22$, $p\leq0.001$) and grower-finisher ($r=0.67$, $p\leq0.001$) with pre-slaughter weight (Table 2.3). As expected, a negative relationship was observed between the ADGs in these two phases with slaughter age. ADG of pigs during these two phases, nursery ($r=-0.18$, $p\leq0.01$) and grower-finisher ($r=-0.72$, $p\leq0.001$) were significantly and negatively correlated with slaughter age (Table 2.3). In this perspective, daily weight gains during the grower-finisher phase demonstrated a stronger linear relationship with pre-slaughter weight and slaughter age than the nursery phase. This was most probably due to the fact that grower-finisher phase had a faster growth in absolute terms with a higher ADG compared to nursery phase, and thus a stronger relationship with the pre-slaughter weight and age.

2.3.2 Causes of mortality and treatment across cycle 1 and 2

There was a mortality rate (including euthanized pigs) of 23.8% (212 pigs) across 14 batches with the total population of 893 pigs. Out of these 212 pigs, cycle 1 and 2 contributed 72.6% (154 pigs) and 27.4% (58 pigs) respectively. Cycle 1 and 2 had a mortality range of 3.6 – 56.7%

and 6.8 – 18.7% across batches, respectively. Three batches of pigs in cycle 1 suffered a mortality rate of over 50%, whilst the highest mortality rate in cycle 2 was only 18.7% (Table 2.4). There were probably several reasons that mortality rate improved in cycle 2: it could be due to the setup of the quarantine and acclimatization unit in an independent building, utilization of PCV2 vaccine and strategic mass medication in the feed and water.

In cycle 2, a quarantine and acclimatization unit, CRSAD was established 3.5 km away from the test station. The main aim was to allow sufficient time (3 weeks) for these pigs to adapt to their new environment and to minimize the routes of disease transmission between the different stages of the model (Figure 2.2). In cycle 1, the nursery unit of the test station was used as a quarantine unit, although separated by walls, common areas such as the utility room, pantry and office, nursery unit and grower-finisher unit where disease challenge was taking place were within the same building. Close distance between the two rooms within the same building increased risk of disease transmission that may easily have occurred through multiple routes. Although special biosecurity protocols were implemented, risk of transmission through fomites was unavoidable, especially through the boots and coveralls of technicians. Multiple epidemiological studies have also confirmed the airborne transmission of PRRSV, although the distance to which it may travel was still highly debatable (Mortenson et al., 2002).

High mortality and treatment rates associated with PCVAD in cycle 1 warranted vaccination of PCV2 in the cycle 2. Pigs infected with PCVAD exhibited a range of clinical signs including wasting syndrome, respiratory distress, diarrhea, jaundice etc. Pigs were most susceptible to PCVAD between eight and fifteen weeks of age which coincides with rapid decline of maternally derived antibodies in the pigs resulting in high post-weaning mortality (Lukert, 1999; Alarcon at al., 2010). This has coincided with our observation that 24% of dead pigs in cycle 1

were in the highest risk age window and they were presented as severe wasting syndrome or 'unthrifty' condition, the leading cause of death in cycle 1 (Table 2.4). Mean age and weight of mortality in cycle 1 were 78.3 days old and 17.9 kg respectively, both younger and lighter in body weight than pigs dead in cycle 2 (Table 2.2). In the grower-finisher phase, PCVAD further incurred production losses through the reduction of ADG and increased FCR (Alarcon et al., 2010). This was also observed in our challenge model, a 5 kg difference in slaughter weight of pigs between cycle 1 and 2 at the same slaughter age, 181 days (Table 2.2).

Water medication and pulse antibiotic medication in feed were also administered during nursery and grower-finisher phases respectively, starting in cycle 2 to further control disease morbidity and reduce mortality. These few strategic and targeted measures were successful in reducing mortality percentage from 34.9% in cycle 1 to 12.9% in cycle 2 (Table 2.4).

Respiratory disease proved to have caused the highest percentage of mortality, 27.6% of total dead in cycle 2 (Table 2.4). Mortality caused by respiratory disease can be associated with multiple factors, the origin could be due to infectious diseases and/or barn management-related environmental issues (dust, ammonia, temperature, humidity etc.). Porcine respiratory disease complex (PRDC) is a serious health problem affecting grower-finisher pigs typically around 16 – 22 weeks of age (Kim et al., 2003). Clinical signs of PRDC include coughing, dyspnoea, lethargy, anorexia, fever, slow growth and poor feed efficiency (Halbur, 1998; Thacker, 2001). It is the description of respiratory disease in swine resulting from a combination of infectious agents (Halbur, 1998; Thacker, 2001). Pathogens involved in PRDC could be either of viral or bacterial origin, but it was often a synergistic combination of both (Table 2.5) (Halbur, 1998; Thacker, 2001; Kim et al., 2003). The two most common pathogens isolated from pigs with PRDC are *Mycoplasma hyopneumoiae* and PRRSV, both of which were present within the challenge model

(Dee, 1996; Thacker et al., 1999). Severity of PRDC and its impact on mortality and morbidity rates often depended on the degree of opportunistic secondary bacterial co-infection.

Besides mortality caused by blood sampling and sudden death, animals that experienced arthritis, diarrhoea, meningitis, lameness, paralysis, anemia that were unresponsive to treatment, rectal prolapse, fighting, bone fracture, and herniation were also euthanized (Table 2.4).

Cycle 2 had a lower injectable antibiotic treatment (877 treatments) than in cycle 1 (1134 treatments) (Table 2.4). Most of these treatments were targeted for respiratory distress and diarrhea (Table 2.4). Unlike diseases that affect the respiratory system, diarrheic animals usually respond well to prompt antibiotic injection and rarely died from this complication. The most common diarrhea in the test station was caused by *Brachyspira hampsonii*. It is a strongly beta-hemolytic spirochete that resembles swine dysentery (caused by *Brachyspira hyodysenteriae*). Pathogenic potential of *Brachyspira hampsonii* have been extensively studied and confirmed in recent years as an emerging novel species in the United States (Burrough et al., 2012; Chander et al., 2012).

Treatment for sarcoptic mange constituted 26.5% of the total treatment in cycle 1 (Table 2.4). It is a type of parasitic disease of the skin caused by *Sarcoptes scabiei.* Sarcoptic mange caused intense and persistent skin irritation and significantly depressed growth rate and feed efficiency of pigs (Reichard and Thomas, 2012). These mites spread by direct contact through infected pigs and contaminated fomites and die within days off the skin of pigs. There was no treatment record of sarcoptic mange in cycle 2 which indicated a good control and eradication from the barn.

2.4 Conclusion

In a conclusion, NDCM is an unusual challenge model that provides an environment where multiplier pigs were exposed to natural infection and subsequently succumbed to the various diseases as evidenced in the analyses in this chapter. The large number of animals involved provided high statistical strength to the on-going studies under this project. It was also the first challenge model that exposed pigs from a high health environment (multiplication farm) to a cocktail of commercially common swine pathogens. Common stressors that closely resembled a commercial barn, which included barn management, stocking density, air quality, housing design, feed types, feeding system etc. were all present in NDCM. All these features enabled a collection of valuable phenotypes, both performance data and biological samples that were vital in characterizing the disease resilience trait in pigs.

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Table 2.1 Biological samples collected during the natural disease challenge model

Bleed	Approximate age of pig (days)	Cycle 1 Location	Cycle 2 Location	Biological samples
1	26	Nursery	CRSAD	EDTA[1], Tempus[2], Serum[3]
2	40	Nursery	CRSAD	Serum, fecal swab
3	54	Nursery	Nursery	EDTA, Tempus, fecal swab
4	82	Grower-finisher	Grower-finisher	EDTA, Tempus, fecal swab
5	96	Grower-finisher	Grower-finisher	Serum, Tempus for sick and healthy pigs (6 each, pen-matched)
6	105	Grower-finisher	-	Serum for seeder pigs (12 weeks post-entry)

[1]EDTA = Whole blood collected in Ethylene Diamine Triacetic Acid, lavender top tubes

[2]Tempus = Whole blood collected in Tempus™ Blood RNA tube (Thermo Fisher Scientific Inc., Wilmington, USA), blue top tubes

[3]Serum = Serum collection using red top tube

Table 2.2 Means and standard errors of performance variables by batches of pig.

Cycle	Batch	N	Mortality		Entry		Mortality		Slaughter	
			N	%	Age (days)	Weight (kg)	Age (days)	Weight (kg)	Age (days)	Weight (kg)
1	1	56	2	3.57	20.89±0.09	7.43±0.13	26.5±0.5	9.0±1.5	176.43±1.41	118.81±1.51
	2	69	17	24.64	22.48±0.44	5.54±0.12	90.53±10.15	16.72±2.83	179.04±1.44	114.38±1.54
	3	60	18	30.00	21.41±0.13	6.90±0.13	91.22±5.59	21.61±3.49	188.67±1.60	115.44±1.71
	4	76	42	55.26	20.61±0.17	6.05±0.11	82.57±3.62	20.65±2.41	184.53±1.78	122.65±1.90
	5	60	34	56.67	21.00±0.08	6.81±0.13	77.76±4.52	18.72±3.16	182.92±2.03	118.88±2.18
	6	60	30	50.00	21.00±0.19	6.07±0.13	69.53±3.62	12.5±0.83	185.83±1.89	116.68±2.03
	7	60	11	18.33	18.62±0.18	5.63±0.13	56.73±9.15	16.14±3.45	180.04±1.48	117.05±1.59
	Subtotal	441	154	34.92	20.85±0.10	6.31±0.06	78.29±2.28	17.85±1.14	181.84±0.65	117.45±0.66
2	8	75	14	18.67	20.75±0.14	7.03±0.11	89.71±8.84	35.32±5.25	181.74±1.20	124.20±0.98
	9	60	4	6.78	22.0±0	6.04±0.11	108.75±15.61	38.6±10.58	186.67±0.95	117.00±0.95
	10	75	9	12.16	23.52±0.06	7.57±0.09	102.78±15.59	47.78±11.7	183.21±0.64	124.59±1.34
	11	28	3	10.71	19.86±0.29	6.66±0.15	117.67±9.33	51.67±4.41	161.86±0.29	99.4±3.22
	12	77	11	14.29	19.71±0.15	7.06±0.11	121.09±9.21	58.91±7.86	183.68±0.18	128.29±1.59
	13	62	6	9.84	20.79±0.12	6.69±0.09	100.57±12.85	47.71±8.46	175.71±1.32	127.68±1.34
	14	75	11	14.67	21.97±0.23	5.63±0.18	104.18±11.39	31.36±6.50	179.63±0.90	115.60±1.20
	Subtotal	452	58	12.92	21.35±0.08	6.69±0.06	104.25±4.60	43.33±3.25	181.79±0.41*	122.89±0.64*
Total		893	212	23.82	21.10±0.07	6.50±0.04	85.48±2.23	25.02±1.46	181.81±0.37*	120.48±0.47*

*Cycles' averages of slaughter age and weight did not include batch 11

Table 2.3 Performance phenotypic correlations among average daily gain (ADG) in nursery, grower-finisher and weaning-finisher, pre-slaughter age and weight, entry age and weight. Pearson correlation was used to estimate the correlations. Number in brackets are probability values reported as $p \leq 0.05$.

Traits	Entry age	Entry weight	Slaughter age	Pre-slaughter weight	ADG (nursery)	ADG (grower-finisher)	ADG (weaning-finisher)
Entry age	1	0.18(0.0001)	0.03(0.60)	0.04(0.53)	0.26(<0.0001)	0.04(0.39)	0.04(0.36)
Entry weight		1	-0.15(0.01)	0.21(0.0003)	0.38(<0.0001)	0.06(0.22)	0.09(0.06)
Slaughter age			1	-0.19(0.0012)	-0.18(0.0023)	-0.72(<0.0001)	-0.74(<0.0001)
Pre-slaughter weight				1	0.22(0.0002)	0.67(<0.0001)	0.68(<0.0001)
ADG (nursery)					1	0.09(0.05)	0.15(0.002)
ADG (grower-finisher)						1	0.99(<0.0001)
ADG (weaning-finisher)							1

Table 2.4 Causes of mortality and treatment of pigs in all the fourteen batches of pigs in cycle 1 and 2.

Causes	Cycle 1				Cycle 2			
	Mortality		Treatment		Mortality		Treatment	
	N	%	N	%	N	%	N	%
Slaughter	287	65.08	-	-	394	87.75	-	-
Unthrifty[1]	106	24.04	50	4.41	13	2.86	14	1.60
Sarcoptic mange	-	-	301	26.54	-	-	-	-
Others	10	2.27	10	0.88	3	0.66	7	0.77
Blood sampling	9	2.04	-	-	3	0.66	-	-
Respiratory distress	7	1.59	304	26.81	16	3.56	357	40.71
Sudden death	6	1.36	-	-	11	2.45	-	-
Arthritis	3	0.68	40	3.53	4	0.89	77	8.78
Diarrhoea	3	0.68	336	29.63	3	0.66	331	37.74
Meningitis	3	0.68	10	0.88	-	-	5	0.57
Rectal prolapse	2	0.45	1	0.09	2	0.45	1	0.11
Fighting	1	0.23	-	-	1	0.22	-	-
Lameness	-	-	78	6.88	1	0.22	71	8.10
Bone fracture	1	0.23	-	-	-	-	-	-
Herniation	1	0.23	-	-	1	0.22	-	-
Exudative epidermatitis	-	-	4	0.35	-	-	14	1.60
Paralysis	1	0.23	-	-	-	-	-	-
Anemia	1	0.23	-	-	-	-	-	-
Total	441	100	1134	100	452	100	877	100

[1] No specific clinical signs, generalized loss of condition

Table 2.5 Types of pathogens involved in porcine respiratory disease complex (PRDC) in swine

Viral	Bacterial
PRRSV Swine Influenza virus Circovirus (PCV2)	*Mycoplasma hyopneumoiae* *Haemophilus parasuis* *Pasteurella multocida* *Actinobacillus pleuropneumoniae*

Figure 2.1 Pig flow in cycle 1: from multiplier farms to CDPQ test station and timing of blood sampling; clean (green) and disease challenge (red) environments were color coded.

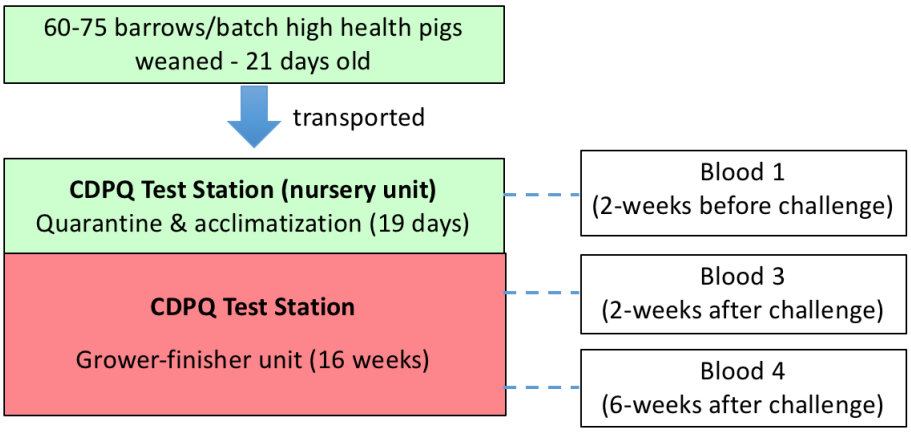


Figure 2.2 Pig flow in cycle 2: from multiplier farms to CRSAD (quarantine and acclimatization unit) and subsequently CDPQ test station and timing of blood sampling; clean (green) and disease challenge (red) environments were color coded.

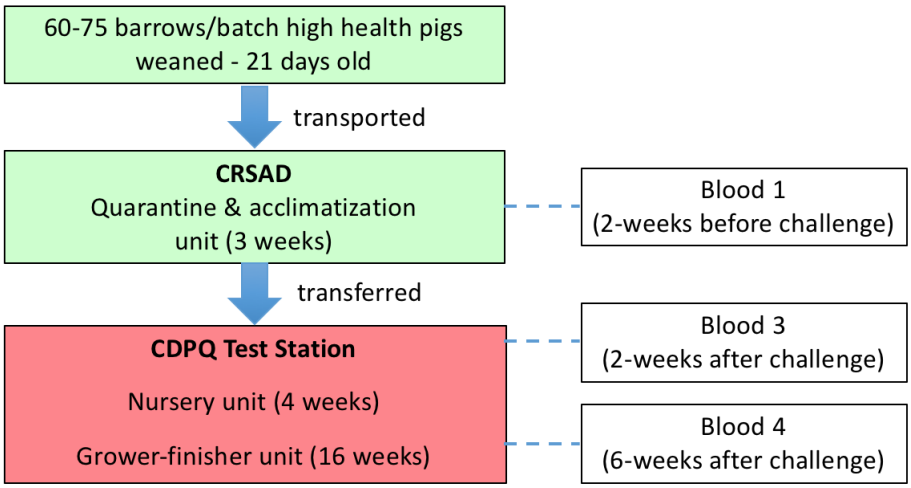
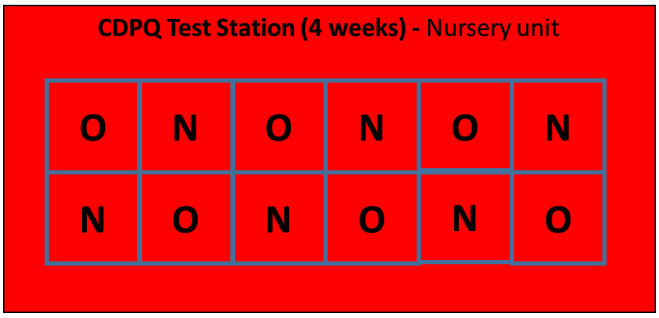


Figure 2.3 Nursery unit arrangement in cycle 2: alternating pens between new (N) and old (O) batches of pigs ran in a continuous flow; each square denoted a nursery pen, disease challenge environment in CDPQ nursery unit was shaded red.



Chapter 3. Hematological Characteristics of Disease Resilient Pigs in a Natural Challenge Model

3.1 Introduction

Hematological testing or complete blood count (CBC) is a common diagnostic test in human and veterinary medicine for routine check of health status and disease diagnosis. CBC measures the concentration of three main cell types in the blood, red blood cells (RBC), white blood cells (WBC), and platelets. It also provides a measure of related traits calculated from or related to these three main cell types, such as hemoglobin, hematocrit, reticulocyte number and numbers for five WBC differential count by cell types (neutrophil, lymphocyte, monocyte, eosinophil and basophil). CBC can be used by clinicians to monitor disease progression, assess bone marrow function, determine the types of anemia etc.

Immune responsiveness has long been proposed as an indirect indicator of disease resistance in animals (Biozzi et al., 1980; Gavora and Spencer, 1983; Rothschild, 1989). It is a measurement of an animal's competency in immunity, the defence system to prevent pathogen invasion and replication. Circulating WBC and its differential cell counts measured in whole blood are each a direct measurement of immune response in an animal. White blood cells or leukocytes are divided into granulocytes, which include neutrophils, eosinophils and basophils, and mononuclear cells, which include lymphocytes and monocytes. Neutrophils represent one of the most important cell types in innate response as they migrate into damaged tissue within hours of an invasion for phagocytosis of foreign material and bacteria (Morris, 2002). Neutrophils are often found in an inflammation caused by gram-negative bacteria and septic shock (Morris, 2002; Irmak et al., 2006). Besides natural killer cells that are part of the innate immunity, lymphocytes are predominantly B cells or T cells of adaptive immunity, where B lymphocytes produce antibodies

and T lymphocytes function in cytotoxic immunity. Both types of lymphocyte provide memory cells during the first pathogen encounter that enable the host to mount a quicker response in subsequent encounters.

Besides WBC, CBC measures RBC which are responsible for gaseous exchange by carrying oxygen and carbon dioxide in their heme structure. Related parameters include hematocrit, a measure of hemoglobin content, mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC) etc. These parameters allow clinicians to detect presence and severity of anemia and to assess the types of anemia that may be due to different biological events that happen within the circulatory systems. Generally, these events could be hemorrhage, hemolysis, nutritional deficiencies, malfunction of bone marrow etc. Platelets, small circulating anucleate cells that are crucial in haemostasis by forming a plug to damaged vasculature and maintaining vascular integrity. Known for their role in thrombotic function, it has become clear in recent years that platelets also play an important role in inflammation and both innate and adaptive immunity (Stoppelaar et al., 2014).

On the other hand, cytokines are key regulators in host defense against pathogens. These proteins are potent immunomodulatory molecules that act as mediators of inflammation and the immune response. Pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-1 beta (IL-1B) and IL-6 are produced by activated macrophages during innate immune response following microbial infections and are involved in the up-regulation of inflammatory reactions (Biron and Sen, 2001). In addition, chemokines such as IL-8 play a vital role in directing migration of leukocytes to the site of inflammation (Smart et al., 1994; Bittleman et al., 1995). The production of appropriate amounts of pro-inflammatory cytokines is clearly beneficial in response to infection, however if produced excessively, these cytokines may lead to tissue destruction and

in some cases, septic shock and death. Driven primarily by the cytokines TNF-α and IL-1B produced by monocytes, excessive inflammation in response to an infection that characterizes septic shock significantly increases mortality. Thus, regulatory cytokines such as IL-10 and TGF-β that serve to regulate/resolve inflammation and to promote healing are also being produced. These regulatory cytokines suppress the host's cell-mediated immune response by reducing cell recruitment and downregulation of cytokine production by innate immune cells (Didierlaurent et al., 2007). Yet, an elevated level of IL-10 has been identified as an early sign of PCV2-infected piglets which are immunosuppressed and subsequently developed severe post-weaning multi-systemic syndrome (PMWS) (Stevenson et al., 2006). In adaptive immunity, interferon gamma (IFN-γ) has also been known for its function in inhibiting viral replication. Production of IFN-γ by macrophages and lymphocytes has been suggested to have an inhibitory effect on the replication of PRRSV (Thanawongnuwech et al., 2003). A combined effect of three cytokines, IL-1B, IL-8 and IFN-γ in serum has also been significantly correlated with PRRS virus level and linked to the persistence of PRRS in pigs (Lunney et al., 2010).

In this study, we hypothesized that differences in CBC profiles before and/or after challenge will help identify and explain the ability of resilient pigs to recover from infection and reduce the impact of disease. Furthermore, we look into the innate and adaptive cytokine profiles of selected pigs and hypothesized that susceptible pigs may have higher levels of expression of pro-inflammatory cytokine genes than resilient pigs after disease challenge. Here, our objective is to explore the differences in CBC and cytokine profiles between resilient and susceptible animals before and after a natural disease challenge. In the next chapter, we will use this knowledge of CBC differences in blood collected before disease challenge to develop an algorithm to predict the classification (resilient or susceptible) of an animal based on its growth rate and treatment rate.

3.2 Materials and methods

Experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock (AUP00002227). Pigs were handled in accordance with the guidelines described by the Canadian Council on Animal Care (CCAC, 2009). The animal study was conducted at the Centre de developpement du porc du Quebec inc. (CDPQ) test station and Centre for Research in Animal Science Deschambault (CRSAD) quarantine and acclimatization unit in Deschambault (Quebec City, QC, Canada).

3.2.1 Pigs and natural challenge model

A total of four hundred and forty-two (n=442) high health nursery crossbred barrows of Yorkshire and Landrace lines from multiplier farms were enrolled in this study through PigGen Canada members. The multiplier herds had been followed by a veterinarian for at least six months in order to confirm the absence or effective control of the diseases as mentioned in Chapter 2. These high health multiplier pigs were then introduced into the test station in batches of 60 or 75 pigs every three weeks. In total seven batches of pigs from cycle 2 of the natural disease challenge model were contributed by different PigGen members and transported from their multiplier herds to the testing station. The natural disease challenge model was established at the Deschambault test station of CDPQ in Quebec, Canada. These pigs were weaned at multiplier farms at approximately 21 days of age and transported to CRSAD, a quarantine and acclimatization unit for 3 weeks. Then, they were introduced into the nursery unit of the natural disease challenge test station for 21 days, during which they were placed in alternate pens of pigs from the previous batch for a week to facilitate disease transmission. After one week, pigs from the previous batch were moved to the grower-finisher unit within the same test station building until slaughtering age or

weight. Pigs of the current batch remained in the nursery unit for another two weeks in which they were comingled with pigs from a later batch in the final week to allow disease transmission and the process was repeated for every batch. Pulse medication in the feed and injectable antibiotics were administered to control disease morbidity and mortality in the test station. Nursery and grower-finisher units were run in continuous flow for disease retention and transmission.

The natural disease challenge model has already been described in detail in chapter 2 of this thesis.

3.2.2 Sample collection

Peripheral blood was collected at 2-weeks pre-exposure (Blood (B) 1) at 26-days of age, and 2 (B3) and 6 (B4) weeks post exposure at 54 and 82 days of age via jugular venipuncture. B1, B3 and B4 were the numbering of blood samples collected from the natural disease challenge model for measurement of CBC. Blood was drawn into EDTA tubes (Cat. No. 10213570; Thermo Fisher Scientific Inc., Wilmington, USA) for complete blood count (CBC) analysis and Tempus^{TM} Blood RNA tubes for RNA extraction, according to the manufacturer's protocols (Cat. No. 4342792; Thermo Fisher Scientific Inc., Wilmington, USA). These samples were then packaged and sent by overnight courier to University of Alberta. Out of 21 shipments, 95.2% (20) and 4.76% (1) arrived within 24 and 48 hours respectively. A CBC analysis was performed immediately upon receiving the sample at the U of Alberta. This was performed using the Siemens Advia 2120 (Siemens Healthcare Diagnostics, Deerfield, IL, USA) for blood from each pig at B1, B3 and B4 (including RBC, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), and platelet, total WBC, lymphocyte, monocyte, neutrophil, eosinophil

and basophil counts) (Table 3.1). A swine specific standard was used for comparison. Based on the results of hematology, sixteen animals (eight from each group of resilient and susceptible animals) were selected for a further cytokine gene expression experiment using a Tempus^{TM} Blood RNA tube.

3.2.3 Disease resilience classification

Animals were classified into resilient and susceptible groups using growth rate and treatment number. Growth rate was generated using linear regression based on the body weight and age of pigs recorded in the test station at three-week intervals. Treatment number per animal was calculated by the addition of number of times injectable antibiotic was administered to the animal throughout the production cycle. Treatment of illness caused by physical trauma, or due to blood collection, or any other causes that were not related to infectious diseases were not included in the calculation of treatment number.

Means of growth rate were determined. Resilient animals (RES) were classified with growth rate above the mean value of all the animals and one treatment or less throughout the production. In other words, RES was defined as an animal with higher than average growth rate and lower treatment number as indicated as quadrant I in Figure 3.1. Animals in quadrants II, III and IV were then classified as susceptible animals (SUS) and these groups included dead and euthanized animals. Causes of death were screened to include only those caused by infectious diseases. Animals that died due to causes that were identified as non-infectious, which included traumatic injuries and death during blood collection, were excluded from this analysis.

3.2.4 Selection of animals for cytokine gene expression

Sixteen animals from batch 14 were selected for subsequent cytokine gene expression analysis, with eight animals from each RES and SUS group. Selected animals experienced leukocytosis (WBC > 22,000 cells/ul) in B4 (Table 3.2). RES animals were found to have high neutrophil-lymphocyte ratio (NLR) in B1 and low NLR in B4, which was the direct opposite for SUS animals (Table 3.2). These criteria were chosen to explore the differences in gene expression of cytokines involved in innate and adaptive immunity between RES and SUS animals during disease challenge period. The gene expression of six cytokine genes, *TNF-α, IL-1B, IL-6, IL-8, IFN-γ* and *IL-10* were investigated.

3.2.5 RNA purification and qPCR

All peripheral blood samples in Tempus^{TM} Blood RNA tubes were immediately kept frozen at −80 °C until RNA extraction. Isolation of RNA from all samples was simultaneously carried out using the Norgen total RNA purification kit (Norgen Biotek Corporation, Canada) according to the manufacturer's instructions. The RNA concentration and purity of all samples were measured with NanoDrop (ND-1000 Spectrophotometer V3.5, USA). RNA integrity was evaluated using an Agilent 2100 BioAnalyzer and only those with a RNA integrity number greater than 7 were further processed.

Total RNA (1 µg) was reverse transcribed to cDNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. qRT-PCR reactions were performed using 1 µl of cDNA diluted 10x with 9 µl of master mix containing 5 µl SYBR Green Master mix (Promega, Madison, WI), 0.5 µmol of forward primer, 0.5 µmol of reverse primer and water, in a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA). The

amplification conditions are described by Royaee et al (2004). The fluorescence signal generated with SYBR Green I DNA dye was measured during the annealing steps. Specificity of the amplification was confirmed using melting curve analysis. An inter-run calibrator was used, and a standard curve was created for each gene to obtain PCR efficiencies. Relative sample expression levels, RQ values, were calculated using RQ Manager 1.2 software (Applied Biosystems), and were expressed relative to endogenous control *RPL32*; *HPRT* was present as an additional internal PCR control for accurate and reliable normalization of qPCR results. Data were corrected for run variability and presented as mean + SEM of RQ values. The GenBank accession numbers, sequences of forward and reverse primers, annealing temperature and fragment sizes of six cytokines mRNAs (*TNF-α, IL-1B, IL-6, IL-8, IFN-γ and IL-10*), *RPL32* and *HPRT* were as previously described (Dawson et al., 2004; Royaee et al., 2004; Sipos et al., 2004; Knetter et al., 2014).

3.2.6 Statistical analysis

Data were analyzed to determine if any of the CBC traits was associated with the resilient classification. A linear model (R/lm) and least-square means (R/lsmeans) were used (Rstudio Team, 2015). The model included resilience groups (RES and SUS) and batch effects. Pig was considered the experimental unit. In the analysis of CBC data, a total of 27 traits (Table 3.1) were analysed. To correct for the multiple testing, the final P values were converted to FDR (q values) using the method of Benjamini and Hochberg (1995), and genes with $q < 0.1$ were declared to be significantly different. Group values for each trait were reported as mean + SEM.

3.3 Results

3.3.1 Pig performance between groups

Using the classification illustrated above, 118 animals were classified as RES, and 231 and 48 live and dead animals were classified as SUS respectively. RES animals were growing with an average growth rate of 837.0 ± 43.7 g/day (grams per day) and far exceeded the growth rate of SUS 722.9 ± 125.4 g/day (Table 3.3). SUS also received higher frequency of antibiotic injections, 2.17 ± 1.5 times compared with 0.58 ± 0.5 times for the RES group during the production cycle. RES also reached higher slaughter weight in a shorter rearing duration: RES took 178 ± 9.0 days to reach an average market weight of 131.5 ± 7.4 kg, 5.8 days faster than SUS, 184.3 ± 7.6 days with a much lower market weight of 120.0 ± 9.9 kg (Table 3.3).

3.3.2 General trend in hematological profiles

Circulating erythrocyte mass appeared to be in a stable state with a small increasing RBC number and a fairly consistent hematocrit (HCT) across the three sample times (B1, B3 and B4) (Table 3.4). Hemoglobin (Hb) on the other hand dropped consistently from B1 to B4. This was also reflected in several other erythrocyte hemoglobin content traits, mean corpuscular hemoglobin concentration (MCHC) and hemoglobin distribution width (HDW). Reticulocyte counts and percentage were found to be increased from B1 to B3, and highest in B3 (Table 3.4). Platelet count was found to have a similar trend as reticulocytes, and was highest in B3 (Table 3.5). For leukocyte traits, the white blood cell (WBC) pool was expanding across samples, as well as the component cells (neutrophil, lymphocyte, monocyte, eosinophil and basophil) within the WBC pool. NLR had the highest value in B3 (Table 3.6).

3.3.3 Hematological profiles before challenge (B1)

For erythrocyte traits, RES had significantly higher MCV (FDR < 0.05) and a higher Hb with a value close to statistical significance (FDR = 0.05) before challenge (Table 3.4). There was no significant difference between groups in all the platelet traits in B1 (Table 3.5). For leukocyte traits, RES had significantly lower lymphocyte and monocyte counts before challenge (both with FDR < 0.05). RES also had a higher neutrophil count, although the difference was not statistically significant (Table 3.6).

3.3.4 Hematological profiles after challenge (B3 and B4)

After challenge, some of erythrocyte traits were found to differ significantly between the two groups, none of platelet and leukocytes traits were found to be significantly different. Those erythrocyte traits that differed were CHCM, RDW, HDW, and reticulocyte count, all with FDR < 0.05 and reticulocyte percentage (FDR = 0.051) in B3; MCH, MCHC and RDW with FDR < 0.05 in B4 (Table 3.4).

3.3.5 Leukocyte & platelet profiles of the selected sixteen animals

In B1, RES pigs had significantly higher counts for total leukocytes, neutrophils and lymphocytes than SUS pigs even before the disease challenge occurred ($p < 0.05$) (Figure 3.2). In B4, SUS pigs had a trend towards a higher leukocyte count ($p = 0.07$) (Figure 3.2).

In B4, apparently, both groups had a significant difference in neutrophil and lymphocyte counts ($p < 0.05$) where neutrophil counts were significantly higher in SUS pigs and lymphocyte counts were significantly higher in RES pigs (Figure 3.2). These differences subsequently translate to a significantly higher NLR in SUS pigs in B4 (Figure 3.2).

3.3.6 Gene expression of cytokine genes of innate and adaptive immunity of the selected sixteen animals

To further characterize the response following disease challenge (B3 and B4), we conducted gene expression analysis of several cytokine genes in whole blood samples. Comparison of cytokine expression before (B1) and after challenge (B3 and B4) revealed that RES and SUS pigs had an up-regulation of expression of all the cytokine genes measured in B3 (Figure 3.3). All these cytokine genes were down-regulated in B4, except for *IL-10* in SUS pigs, and *IFN-γ* in both RES and SUS pigs.

In B3, RES pigs were found to express numerically higher levels of *IL-8* and *IL-1B* genes, whilst SUS pigs expressed a numerically higher level of *TNF-α, IL-6, IL-10* and *IFN-γ*. In B4, the gene expression of all the cytokine genes involved in innate immunity except *IL-6* were higher in SUS pigs than RES pigs with a value close to statistical significance, *TNF-α* ($P = 0.08$) and *IL-1B* ($p = 0.09$), although for *IL-8* it was only higher numerically (Figure 3.3). SUS pigs also had a numerically higher gene expression of regulatory cytokine, *IL-10*.

For comparison across sampling time within group, SUS pigs had a significantly higher expression of *TNF-α*, from B1 to B3 ($p < 0.01$), and from B1 to B3 ($p < 0.05$). SUS pigs also had a numerical increase of *IL-1B* from B1 to B3 ($p = 0.01$) and a trend to drop from B3 to B4 ($p = 0.06$). In addition, expression of *IFN-γ* in SUS tended to increase from B1 to B4 ($p = 0.09$).

3.3.7 Correlation of cytokine gene expression, leukocyte and platelet numbers

Correlation coefficients between the traits have been calculated for all the sixteen selected pigs in both RES and SUS groups (Table 3.7). Our data demonstrated moderate correlations between *IL-1B* with *IL-8* ($r=0.68$, $p < 0.05$) and *TNF-α* ($r=0.53$, $p < 0.05$). There was a moderate

and significant correlation between *TNF-α* and neutrophil number ($r=0.31$, $p < 0.05$). IL-10 was also shown to have low to moderate correlation with *IL-8* ($r=0.32$, $p < 0.05$), *TNF-α* ($r=0.47$, $p < 0.05$), neutrophil number ($r=0.45$, $p < 0.05$) and NL ratio ($r=0.40$, $p < 0.05$) (Table 3.7).

3.4 Discussion

3.4.1 Erythrocyte Traits

Both RES and SUS presented a higher reticulocyte count and percentage in B3 and B4 (Table 3.4). This may due to an increased demand for RBC in the body to meet the requirement for maintenance and growth of the body as more oxygen is needed to support energy production at the cellular level. It could possibly be due to an ongoing loss of RBC in both groups, due to subclinical gastrointestinal (GI) hemorrhage or hemolysis. Causes of GI hemorrhage in pigs include salmonellosis, swine dysentery, ileitis and gastric ulcer. Production and release of reticulocyte, a premature form of RBC, into the blood circulation is induced by tissue hypoxia (Haschek et al., 2013). An increase in reticulocyte count in B3 may be due to redistribution or sequestration of RBC with no involvement of bone marrow (Haschek et al., 2013). Under this condition, RBC, reticulocytes and platelets may transiently increase secondarily to splenic contraction that would be a result of an increased stress level, physical restraint or physical activity (Haschek et al., 2013). This was a plausible explanation as pigs were restrained for venipuncture and could easily become excited, agitated and stressed during the process. Higher stress level in B3 could be associated with an increased pathogen challenge or social aggression in the barn environment.

On a larger scale, increase in reticulocyte number could be due to a loss, destruction or consumption of circulating RBC. A significantly higher level of reticulocytes in SUS in B3 could

be due to a moderate erythrocyte regeneration response with the involvement of bone marrow (Table 3.4). This response is generated to compensate for a loss of circulating RBC that may be due to hemorrhages (internally or externally), hemolysis or a shortened RBC half-life in the circulation caused by anemia of inflammation (Braunstein, 2016).

Although there were no obvious signs of anemia after assessing the group averages of Hb, HCT and RBC, there was a reduction of hemoglobin concentrations from B1 to B4 for both groups. This could be due to the changes in hydration status where expansion of blood volume may reduce the hemoglobin concentration. The SUS group also had a lower hemoglobin than RES group in all three blood samples, especially in B1 (FDR=0.05) with a value close to significance level (Table 3.4). Lower level in B1 could possibly be due to the timing of iron injection administered in the multiplication farm.

3.4.2 Leukocyte Traits

Good evidence of disease challenge in B3 and B4 can be found by the expanding pool of WBC in both groups of animals (Table 3.6). It could be from the maturation of WBC pool in B3 in the reference range of 11 – 22 x 10^3/µL (Thorn, 2010). However, WBC level of both groups in B4 were on the verge of exceeding the suggested reference range and being classified as leukocytosis (Table 3.6). This was most likely due to immune activation elicited by the increasing disease challenge in the test station when pigs were moved from clean quarantine unit to the contaminated nursery and grower-finisher unit. SUS had numerically higher WBC level in all three blood samples compared to RES suggesting a higher immune activation.

Neutrophil-lymphocyte ratio (NLR) was defined as the number of neutrophils divided by the number of lymphocytes. NLR has been correlated with stress in animals (Puppe et al., 1997).

The highest level of NLR was detected in B3 that may suggest B3 as the most stressful period for these pigs (Table 3.6). Moving pigs into a bigger nursery pen with a higher stocking density and changing of creep feed to nursery feed can also cause social and dietary or management stresses. Unfamiliarity as a result of mixing pigs from different pens may lead to aggression in the process of establishing social hierarchy and sometimes end up with serious injury (Meese and Ewbank, 1972; Pitts et al., 2000). The underlying mechanism that raised NLR level in these scenarios could be due to the demargination of granulocytes, predominantly neutrophils from vascular endothelium in response to the release of hormones induced by stress and excitement (Haschek et al., 2013). This mechanism can rapidly increase circulating neutrophils that typically are not accompanied by leukocytosis.

NLR has also been associated with inflammation, and in human medicine it has been developed as a prognostic indicator of cardiovascular disease as it is associated with chronic low grade inflammation (Imtiaz et al., 2012). In B3 and B4, these pigs were exposed to multiple swine pathogens for the first time after spending three weeks in a quarantine unit. Porcine reproductive and respiratory syndrome virus (PRRSV) has been routinely monitored and confirmed to be circulating within the test station by post mortem and polymerase chain reaction. PRRSV has been reported to increase NLR by increasing neutrophil count, and delaying cell mediated immunity by reducing lymphocyte count in infected pigs (Che et al., 2014). Physical injuries such as ear and tail biting, cuts or abrasion on skin that leads to bacterial infection and inflammation may also raise NLR in the circulation. An example of a septicemia causing pathogen is *Streptococcus suis*, commonly introduced through skin wounds due to fighting (McCaw, 1993), this pathogen was not uncommon in our natural challenge model.

SUS animals had significantly higher lymphocyte and monocyte counts but RES animals had higher neutrophil count in B1 (Table 3.6). Neutrophils belong to the innate immune system which is the first line defense against invading microorganisms. Neutrophils are equipped with antimicrobial proteins, reactive oxygen species (ROS), and neutrophil extracellular traps (NETs) as well as being able to phagocytize pathogens (Amulic et. al, 2012). The ability of neutrophils to kill harmful microorganisms is immediate, and non-specific and previous exposure to these microorganisms was not needed (Kobayashi et al., 2009). Neutrophils play an active role in acute inflammation. This suggests that having a higher proportion of neutrophils in B1 allowed the RES group to react quickly to any pathogen invasion that was usually mild and self-limiting in the clean quarantine unit.

In B4, RES had numerically higher monocyte and lymphocyte counts that may indicate the switching from innate immunity to cell mediated immunity that was more specific and less damaging at the site of inflammation (Table 3.6). SUS had a lower level of neutrophil production in B1, and in B3 and B4 they were numerically higher than RES. Persistently increasing neutrophil level in SUS may lead to a higher inflammatory state and collateral damage to host tissue at the site of inflammation. Resolution of inflammation was promoted with the recruitment of monocytes and blocking of neutrophils which was observed in RES animals in B4.

3.4.3 Platelet Traits

Platelet count was highest in B3 (Table 3.5). SUS animals had a higher level of platelets in all three blood samples compared to RES (Table 3.5). Conventionally it was thought that platelets mainly play an important role in haemostasis. But in recent years, platelets were also found to be involved in inflammation and associated with both innate and adaptive immunity (Stoppelaar et

al., 2014). Activation of platelets was known to release a-granule containing soluble inflammatory mediators such as IL-1B, MIP-1a, RANTES and PF4. These mediators had a wide range of interactions with immune cells such as monocytes, neutrophils and macrophages and the recruitment of these cells to the endothelium by the release of pro-inflammatory cytokines that induce phagocytosis and chemotaxis, promote migration and formation of bridges between leukocytes and endothelium (Thomas & Storey, 2014). Therefore, the higher platelet concentration in SUS animals could very well be correlated with the extent of inflammation and it could also be related to its function in blood coagulation.

3.4.4 Leukocyte and platelet profiles and gene expression of immunity cytokines in selected RES and SUS animals

These pigs were selected based on the high level of leukocyte number after challenge. This presented an opportunity to explore the possible different defense mechanisms between groups. It was observed that RES pigs had significantly higher counts for total leukocytes ($P < 0.05$), neutrophils ($P < 0.05$) and lymphocytes ($P < 0.05$) even before the disease challenge (B1) occurred (Figure 3.2). However, there was no difference in terms of the expression levels of cytokine genes in B1. As all these pigs were chosen from the same batch, they were assumed to be exposed to the same stress factors such as transportation, handling procedure, and a week of acclimatization period in the quarantine unit. A possible mechanism that could cause the differences in differential leukocyte count was RES pigs could be innately and genetically equipped to produce a higher total leukocyte, neutrophil and lymphocyte counts even without the presence of disease challenge. In an alternative mechanism, the regulation of immune system in RES pigs may be able to react more

to environmental stimuli compared to SUS pigs, thus resulting in a higher circulating levels of immune cell types.

In the first exposure to disease challenge in B3, both RES and SUS pigs experienced an up-regulation of all the cytokine genes involved in innate and adaptive immunity (Figure 3.3). This included cytokines such as the *TNF-α, IL1B, IL-6* and *IL-8* that are pro-inflammatory. RES pigs had a much higher *IL-8* and *IL1B* expression, and lower *TNF-α* and *IL-6* expression than SUS pigs (Figure 3.3). Our data also demonstrated moderate correlations between *IL-8* and *IL-1B* ($r=0.68$, $p < 0.05$), correlation between *IL-8* and *TNF-α* was however not significant (Table 3.7). TNF-α and IL-1B have been associated with neutrophil infiltration in several animal models (Reeth et al., 2002). TNF-α is a well-known mediator of local events at the site of inflammation that attract and activate inflammatory cells, and increase of microvascular permeability (Adler et al., 1994; Bielefeldet-Othmann et al., 1995). TNF-α and/or IL1B are essential in the initial adhesive reaction of neutrophils to the microvascular endothelium. On the other hand, IL-8 is a major neutrophil chemotactic cytokine that was needed in the directed migration of leukocytes to the site of inflammation (Smart et al., 1994; Bittleman et al., 1995). Higher levels of serum IL-8 have been associated with PRRSV resistance in pigs, and these pigs were able to clear the infection before it developed into a persistent infection (Petry et al., 2007; Lunney et al., 2010). Although there was an up-regulation in all the innate cytokines in B3 for both groups, higher expression level of *IL-1B* and *IL-8* in RES pigs may have enabled a more effective migration of leukocyte and clearance of pathogens.

SUS pigs were found to have numerically higher expression of regulatory cytokine, *IL-10* in all three blood samplings. This indicated that SUS pigs were dealing with chronic inflammatory challenge. Some swine diseases are also known to elevate IL-10 this includes PRRS and PCV2

(Hasslung et al., 2005; Stevenson et al., 2006). IL-10 mediated suppression of T-cell activity was also well documented and has been suggested to be one of the causes of viral persistence (Marin-Serrano et al., 2006; Orsilles et al., 2006; Brooks et al., 2006). This may have explained the lower level of lymphocyte count in B4 ($P<0.05$) and possible viral infection/disease in SUS pigs during that time (Figure 3.3).

In B4, cytokine gene expression of RES pigs revealed a decrease across *TNF-α*, *IL-1B* and *IL-8* genes ($p = 0.06$) (Figure 3.3). This could have indicated resolution of inflammation, and cessation of recruitment and activation of neutrophils in RES pigs. On the other hand, there was a steady increase of *IFN-γ* in RES pigs (Figure 3.3). IFN-γ secretion was widely used for the assessment of antigen-specific Th1 immune responses in swine (Yuan et al., 2008). Expression of *IFN-γ* by macrophages and lymphocytes was suggested to have an inhibitory effect on the replication of viral agents such as PRRSV (Thanawongnuwech et al., 2003). This was reflected in the increase of lymphocyte number which may also be due to the lower level of IL-10, which could be the development of Th1 immune response in RES pigs. On the contrary, higher levels of *IL-8*, *TNF-α* ($P=0.08$) and *IL-1B* (0.09) were detected in SUS pigs in B4. This seemed to result in a persistently high neutrophil number in B4 ($P < 0.05$) in SUS pigs (mean of 21.4 x 10^3/μL), where the reference range is 0.8 – 13 x 10^3/μL (Cooper et al., 2014) (Figure 3.2). The resulting proportion of neutrophils and lymphocytes was significantly lower ($p < 0.05$) as NLR in RES compared to SUS, which has been associated with a lower stress and inflammatory level in the animals.

3.5 Conclusion

In conclusion, CBC has provided us with a snapshot of the health status of these pigs at several critical time points. The hematological profile has revealed that RES pigs may have a

higher level of innate immunity before disease challenge that enables them to react more quickly against invading microorganisms. Whilst, SUS pigs have a persistently higher level of neutrophils after disease challenge that may cause excessive inflammation and tissue damage that have an impact on their growth rate. Here, RBC, platelet and their related traits did not provide any insights on the resilience trait.

Differences in leukocyte profiles in both RES and SUS pigs were supported by the results obtained from cytokine gene expression analysis. Based on the cytokine gene expression results of the selected pigs, RES pigs can be associated with a higher *IL-8* and *IL-1B* gene expression 2 weeks post challenge (B3); lower *TNF-α, IL1B, IL-8* and *IL-10* expression levels, and higher *IFN-γ* 6 weeks post challenge (B4). This was suggestive of resolution of inflammation in B3 and B4 and development of adaptive immunity in B4. On the other hand, SUS pigs had persistently high *IL-10, IFN-γ, TNF-α, IL-8* and *IL-1B* throughout the blood samplings that may be suggestive of a persistent chronic inflammatory response.

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Table 3.1 Red blood cells, white blood cells, platelet and related traits calculated from or related to these three main cell types measured in complete blood count from whole blood collected in B1, B3 and B4.

Complete blood count		
Red blood cells[1]	White blood cells[2]	Platelet[3]
RBC count, $10^6/\mu L$	WBC count, $10^3/\mu L$	Platelet, $10^3/\mu L$
Hemoglobin, g/dL	Neutrophils, $10^3/\mu L$	MPV, fL
Hematocrit, %	Lymphocytes, $10^3/\mu L$	PDW
MCV, fL	Monocytes, $10^3/\mu L$	PCT
MCH, pg	Eosinophils, $10^3/\mu L$	
MCHC, g/L	Basophils, $10^3/\mu L$	
CHCM	N:L ratio	
RDW, %	Neutrophil, %	
HDW	Lymphocyte, %	
Reticulocyte count, $10^3/\mu L$	Monocytes, %	
Reticulocyte, %	Eosinophil, %	
	Basophil, %	

[1]RBC = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCHC = mean corpuscular hemoglobin concentration; CHCM = Cell hemoglobin concentration mean; RDW = RBC distribution width; HDW = Hemoglobin distribution width.
[2]WBC = white blood cells; N:L ratio = Neutrophil:Lymphocyte ratio.
[3]MPV = mean platelet volume; PDW = platelet distribution width; PCT = plateletocrit.

Table 3.2 Least squares means and standard errors of leukocyte traits for Resilient (n=8) and Susceptible groups (n=8) in batch 14.

Group	Blood 1[1]		Blood 3[1]		Blood 4[1]	
	RES	SUS	RES	SUS	RES	SUS
WBC[2]	13.23±0.45	8.55±0.22*	21.03±0.52	21.11±0.54	26.84±0.93	33.45±0.85
NLR[2]	1.32±0.01	1.08±0.05	1.95±0.15	1.96±0.10	1.09±0.04	2.44±0.14*

[1]Blood 1 = blood sample taken 2-weeks before challenge; Blood 3 = blood sample taken 2-weeks after challenge; Blood 4 = blood sample taken 6-weeks after challenge.
[2]WBC = White blood cells ($x10^3$ cells/ul); NLR = neutrophil-lymphocyte ratio.
* indicates $P < 0.05$

Table 3.3 Pig performance between RES and SUS groups

Performance Traits	Groups	
	Resilient	Susceptible
Number of pigs	118	231 (48[#])
Growth Rate (g/day)[1]	837.0 ± 43.7	722.9 ± 125.4*
Treatment number per animal[1]	0.58 ± 0.5	2.17 ± 1.5*
Slaughter weight (kg)[1]	131.5 ± 7.4	120.0 ± 9.9*
Days to market (days)[1]	178.5 ± 9.0	184.3 ± 7.6

[#]48 dead pigs classified under Susceptible group.
[1]Performance traits do not include those from dead animals.
* indicates $P < 0.05$

Table 3.4 Least squares means[1] and standard errors of erythrocyte traits for Resilient (n=118) and Susceptible groups (n=279)

CBC Traits[2]	Blood 1[3]			Blood 3[3]			Blood 4[3]		
	RES[4]	SUS[4]	FDR[5]	RES[4]	SUS[4]	FDR[5]	RES[4]	SUS[4]	FDR[5]
RBC count, $10^6/\mu L$	6.09±0.07	6.11±0.05	0.8554	6.18±0.07	6.28±0.04	0.4491	6.37±0.06	6.32±0.06	0.6805
Hemoglobin, g/dL	120.36±1.29	116.61±0.83	0.0504	109.86±1.42	107.83±1.09	0.4491	108.01±1.31	105.10±1.26	0.2809
Hematocrit, %	0.38±0.004	0.37±0.003	0.1851	0.35±0.005	0.35±0.003	0.5514	0.36±0.01	0.35±0.01	0.4989
MCV, fL	61.94±0.46	60.19±0.33	**0.017***	56.04±0.30	55.87±0.19	0.7656	56.11±0.28	55.81±0.20	0.5514
MCH, pg	20.27±0.45	19.57±0.28	0.4130	13.46±0.69	14.03±0.40	0.6322	12.39±0.69	14.78±0.36	**0.017***
MCHC, g/L	326.18±6.13	323.38±3.58	0.8031	239.41±12.23	251.30±7.09	0.5514	222.62±12.39	265.04±6.39	**0.017***
CHCM	281.41±1.02	281.89±0.77	0.8031	289.13±1.04	285.38±0.74	**0.017***	284.97±1.32	283.09±0.91	0.4491
RDW, %	20.79±0.35	21.74±0.27	0.1040	18.13±0.21	19.10±0.19	**0.017***	18.12±0.12	18.57±0.09	**0.017***
HDW	19.49±0.30	19.96±0.21	0.4166	19.56±0.21	18.74±0.13	**0.017***	18.95±0.19	18.88±0.16	0.8479
Reticulocyte count, $10^3/\mu L$	155.75±6.63	156.64±4.69	0.9128	192.11±11.27	231.41±9.75	**0.036***	190.96±6.51	178.01±6.27	0.3610
Reticulocyte, %	2.55±0.10	2.57±0.07	0.8902	3.13±0.18	3.71±0.15	0.0505	3.04±0.11	2.88±0.11	0.4989

[1]Least squares means ± SE are from the lsmeans in R.

[2]RBC = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCHC = mean corpuscular hemoglobin concentration; CHCM = Cell hemoglobin concentration mean; RDW = RBC distribution width; HDW = Hemoglobin distribution width.

[3]Blood 1 = blood sample taken 2-weeks before challenge; Blood 3 = blood sample taken 2-weeks after challenge; Blood 4 = blood sample taken 6-weeks after challenge.

[4]RES = Resilient group defined by growth rate of =>0.7615 kg/day and treatment rate of <=1; SUS = Susceptible group defined by these three conditions: growth rate of =>0.7615 kg/day and treatment rate >1; growth rate of <=0.7615 kg/day; dead pigs.

[5]FDR = false discovery rate value, * indicates FDR < 0.05

Table 3.5 Least squares means[1] and standard errors of platelet traits for Resilient (n=118) and Susceptible groups (n=279)

CBC Traits[2]	Blood 1[3]			Blood 3[3]			Blood 4[3]		
	RES[4]	SUS[4]	FDR[5]	RES[4]	SUS[4]	FDR[5]	RES[4]	SUS[4]	FDR[5]
Platelet, $10^3/\mu L$	273.00±17.92	325.63±11.72	0.1768	453.75±17.33	470.24±10.96	0.7383	270.94±13.90	290.71±11.34	0.6518
MPV, fL	13.12±0.34	12.39±0.14	0.2876	12.68±0.14	12.67±0.13	0.9688	12.94±0.14	12.95±0.14	0.9688
PDW	61.79±1.34	62.95±0.76	0.7383	63.60±0.64	62.29±0.36	0.3003	68.18±0.71	68.80±0.55	0.7383
PCT	0.003±0.001	0.003±0.0004	0.7812	0.01±0.0007	0.01±0.0005	0.7812	0.002±0.0004	0.002±0.0003	0.3754

[1]Least squares means ± SE are from the lsmeans in R.
[2]MPV = mean platelet volume; PDW = platelet distribution width; PCT = plateletocrit.
[3]Blood 1 = blood sample taken 2-weeks before challenge; Blood 3 = blood sample taken 2-weeks after challenge; Blood 4 = blood sample taken 6-weeks after challenge.
[4]RES = Resilient group defined by growth rate of =>0.7615 kg/day and treatment rate of <=1; SUS = Susceptible group defined by these three conditions: growth rate of =>0.7615 kg/day and treatment rate >1; growth rate of <=0.7615 kg/day; dead pigs.
[5]FDR = false discovery rate value, * indicates FDR < 0.05

Table 3.6 Least squares means[1] and standard errors of leukocyte traits for Resilient (n=118) and Susceptible groups (n=279)

CBC Traits[2]	Blood 1[3]			Blood 3[3]			Blood 4[3]		
	RES[4]	SUS[4]	FDR[5]	RES[4]	SUS[4]	FDR[5]	RES[4]	SUS[4]	FDR[5]
WBC count, $10^3/\mu L$	11.17±0.38	11.66±0.22	0.5833	18.79±0.39	19.56±0.35	0.4318	21.74±0.52	22.24±0.42	0.6823
Neutrophils, $10^3/\mu L$	5.30±0.30	5.19±0.17	0.7960	9.36±0.30	9.52±0.28	0.7960	10.22±0.39	10.74±0.33	0.5876
Lymphocytes, $10^3/\mu L$	4.96±0.14	5.51±0.10	**0.038***	6.88±0.20	7.39±0.17	0.4151	9.18±0.29	9.16±0.22	0.9582
Monocytes, $10^3/\mu L$	0.38±0.02	0.45±0.01	**0.038***	1.27±0.07	1.31±0.06	0.7960	1.40±0.06	1.33±0.06	0.6823
Eosinophils, $10^3/\mu L$	0.31±0.02	0.28±0.01	0.4452	0.75±0.10	0.78±0.07	0.7999	0.40±0.02	0.44±0.02	0.4318
Basophils, $10^3/\mu L$	0.05±0.01	0.04±0.002	0.6485	0.12±0.01	0.13±0.01	0.5056	0.30±0.04	0.31±0.02	0.7960
N:L ratio	1.11±0.06	1.00±0.03	0.4151	1.45±0.05	1.53±0.08	0.6823	1.28±0.08	1.31±0.05	0.7960
Neutrophil, %	45.24±1.05	43.03±0.67	0.4151	49.35±1.02	48.06±0.75	0.5876	46.29±1.09	47.04±0.84	0.7875
Lymphocyte, %	46.23±1.04	48.53±0.64	0.4151	37.02±0.89	38.66±0.68	0.4318	42.79±1.05	42.28±0.81	0.7960
Monocytes, %	3.55±0.14	3.90±0.10	0.4151	6.84±0.39	6.58±0.24	0.7708	6.49±0.25	6.04±0.26	0.4921
Eosinophil, %	3.02±0.21	2.59±0.14	0.4151	3.96±0.49	3.75±0.31	0.7960	1.89±0.09	2.06±0.08	0.4452
Basophil, %	0.41±0.04	0.34±0.01	0.4318	0.62±0.03	0.66±0.02	0.6823	1.36±0.14	1.48±0.12	0.7560

[1]Least squares means ± SE are from the lsmeans in R.
[2]WBC = white blood cells; N:L ratio = Neutrophil:Lymphocyte ratio.
[3]Blood 1 = blood sample taken 2-weeks before challenge; Blood 3 = blood sample taken 2-weeks after challenge; Blood 4 = blood sample taken 6-weeks after challenge.
[4]RES = Resilient group defined by growth rate of =>0.7615 kg/day and treatment rate of <=1; SUS = Susceptible group defined by these three conditions: growth rate of =>0.7615 kg/day and treatment rate >1; growth rate of <=0.7615 kg/day; dead pigs.
[5]FDR = false discovery rate value, * indicates FDR < 0.05

Table 3.7 Pearson's correlation coefficients between leukocytes and differential counts, platelet, cytokines of innate and adaptive immunity in sixteen selected pigs in batch 14

	IFN-γ	IL-10	IL-1B	IL-8	TNF-α	WBC[1]	Neut[1]	Lymp[1]	NL ratio[1]	platelet	IL-6
IFN-γ	1	—	—	—	—	—	—	—	—	—	—
IL-10	0.51**	1	—	—	—	—	—	—	—	—	—
IL-1B	0.08	0.23	1	—	—	—	—	—	—	—	—
IL-8	0.03	0.32*	0.68**	1	—	—	—	—	—	—	—
TNF-α	0.08	0.47**	0.53**	0.22	1	—	—	—	—	—	—
WBC[1]	0.47**	0.40**	-0.01	-0.09	0.23	1	—	—	—	—	—
Neut[1]	0.40*	0.45**	0.07	0.03	0.31*	0.95**	1	—	—	—	—
Lymp[1]	0.47**	0.18	-0.19	-0.28	0.01	0.81**	0.60**	1	—	—	—
NL ratio[1]	0.01	0.40*	0.32*	0.33*	0.38*	0.52**	0.73**	-0.06	1	—	—
platelet	-0.01	0.02	0.11	0.04	0.14	-0.01	0.01	-0.02	0.07	1	—
IL-6	0.14	0.12	0.83**	0.60**	0.34*	-0.01	0.03	-0.08	0.20	0.01	1

* indicates $P < 0.05$

** indicates $P < 0.01$

[1]WBC = white blood cells; Neut = Neutrophil; Lymp = Lymphocyte; N:L ratio = Neutrophil:Lymphocyte ratio.

Absolute neutrophil and lymphocyte counts were used to calculate the correlation between neutrophils and lymphocytes with other parameters.

Figure 3.1 Classification of resilience groups using growth rate and treatment number, RES in quadrant I and SUS in quadrants II, III and IV.

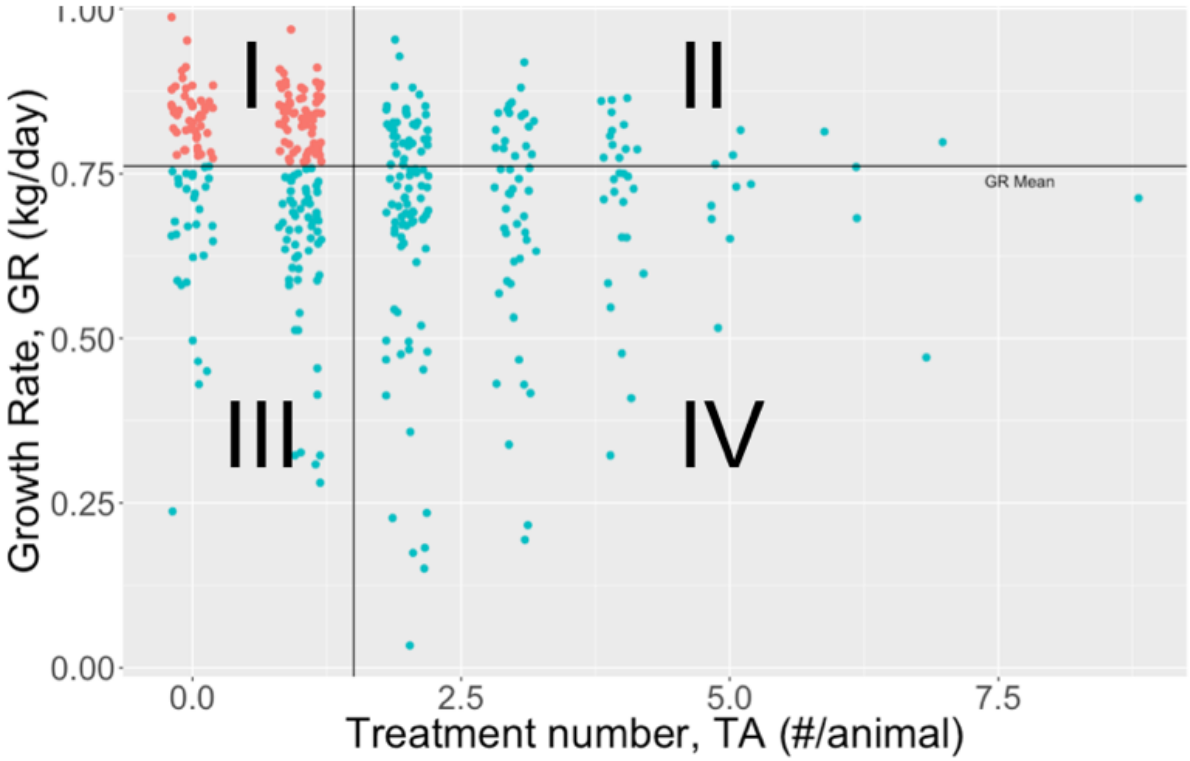


Figure 3.2 White blood cells (WBC) counts and its differential cell types and platelet count of sixteen pigs in batch 14 (eight pigs from each resilient and susceptible group) in response to natural disease challenge. Data were presented as mean + SEM of CBC values; p values were stated for comparisons of group within blood sampling.

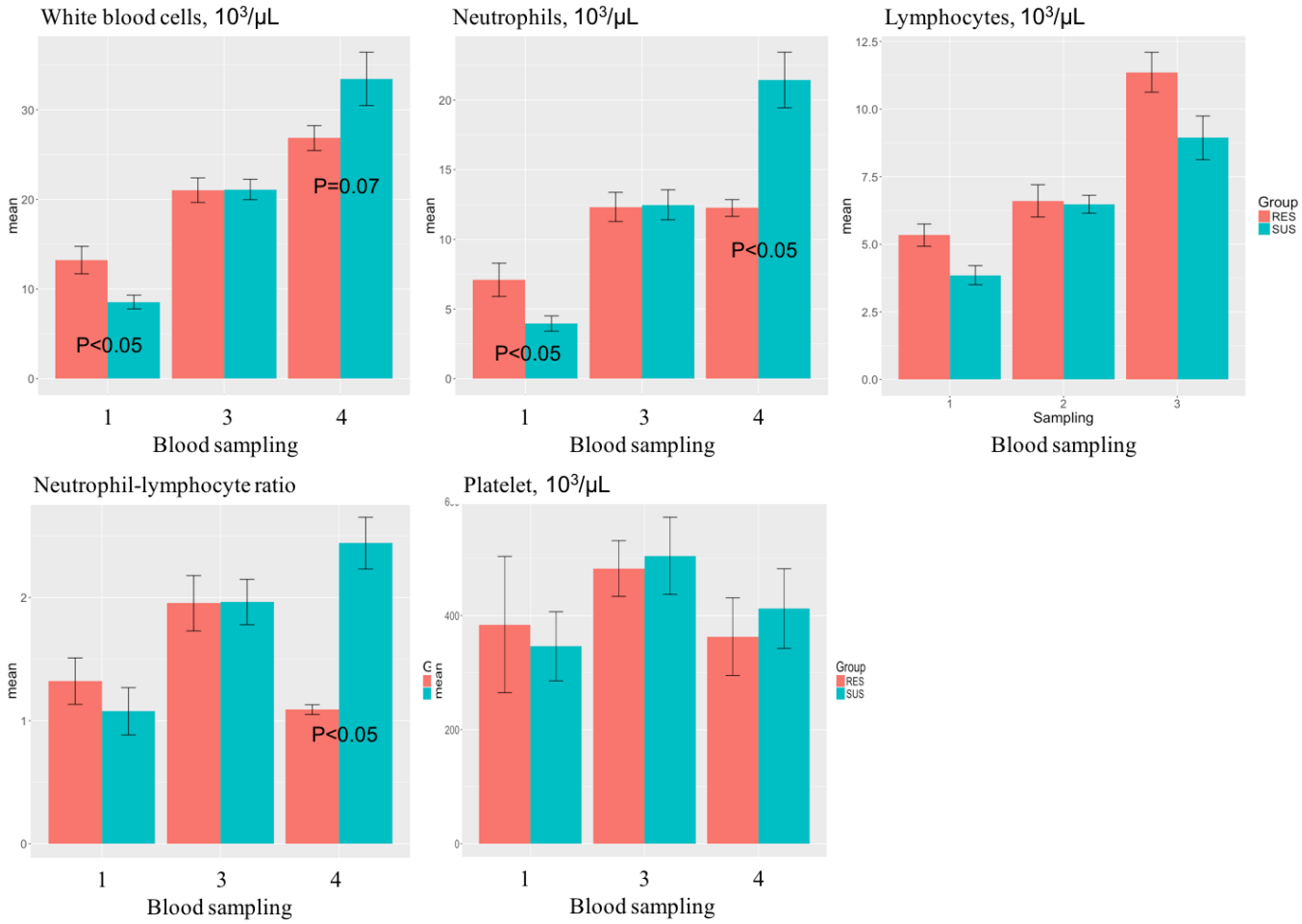
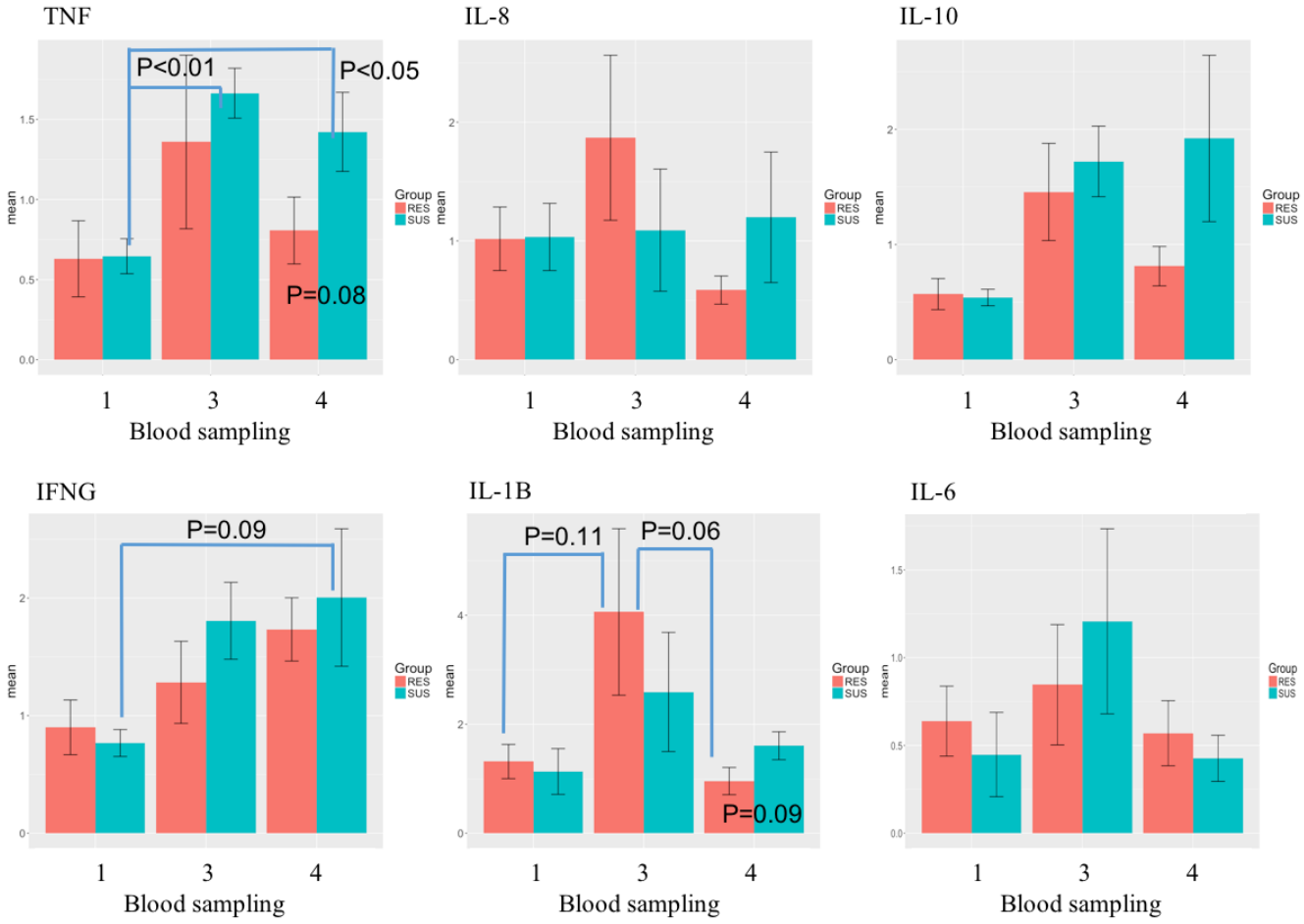


Figure 3.3 Peripheral blood cytokine gene expression of sixteen pigs in batch 14 (eight pigs from each resilient and susceptible group) in response to natural disease challenge. RQ values were calculated and expressed relative to endogenous control RPL32; HPRT was present as an additional internal PCR control. Data were presented as mean + SEM of RQ values; p values were stated for comparisons of group within blood sampling done using student T-test and comparisons across blood sampling within group done using repeated measures.



Chapter 4. Complete Blood Count Based Prediction of Disease Resilient Pigs in a Natural Challenge Model

4.1 Introduction

Disease resilience has been defined as the ability of animal to respond to an immune challenge (i.e. pathogen) in a way that minimizes the impact of disease (Plastow, 2016). Unlike the traits of disease resistance as discussed in Chapter 1, records of pathogen burden of infected animals are not required, which is also one of the main obstacles in studies of this type. This is because these phenotypes are difficult and expensive to collect and hence large scale studies are not possible. Instead, an emphasis on the performance of the animal regardless of pathogen load has been deemed a more practical approach to studying resilience (Mulder and Rashidi, 2017). On the other hand, a disease susceptible animal allows establishment and replication of pathogens, so that infection progresses affecting its growth performance.

Studies done by Knetter et al., (2014) and Kommadath et al., (2014) showed that pigs with low and persistent Salmonella shedding in fecal samples exhibited different immune response within the first 2 days of infection and through genomic analyses, several new candidate regulators of Salmonella shedding in pigs were identified even before the beginning of a Salmonella challenge. Andronicos et al., in 2014 also reported the application of complete blood count (CBC) for the classification of *Haemonchus contortus* resilient sheep using a single blood sample regardless of the infection status. These studies support the notion that there is a practical potential of using blood samples taken before a disease challenge in predicting a disease outcome or trait of disease resilience of an animal. The CBC test offers several attractive features that mean it could be easily implemented in the industry if it is proven useful. Although CBC is not routinely done

on herd animals, it is a routine test in veterinary medicine in assessing the general health of an animal. Hence, the cost of the test and process of biological sample (blood sample) collection are tolerable; it does not require complex equipment nor highly skilled technicians to implement.

Such a "classifier system" for medical diagnosis has been researched extensively in human medicine. These computational intelligence techniques help in diagnosis and early detection of heart disease and diabetes that may prevent these diseases from causing further damage to the body (Polat and Günes, 2007; Nahar et al., 2013). Predictions arising from different techniques assist medical experts in diagnosis and decision for treatment options. Principal component analysis (PCA), is a multivariate technique that has been used to extract important information where observations were described by inter-correlated quantitative dependant variables. This method was based on the assumption that most information about classes lies in the direction where variations are the largest (Polat and Günes, 2007). Before classification, PCA was used in dimensionality reduction of a dataset by representing these variables in a new set of orthogonal variables called principal components (PC) (Abdi et al., 2010). Linear regression with stepwise feature selection was then used in the selection of a combination of PCs that best predict the outcome of different classes. Reduction in dimensionality removed redundant data and thus less computational work was required with a smaller dataset. Generally, this leads to an improved generalization ability of a model and gives better accuracy in its prediction (Maldonado et al., 2011; Piramuthu et al., 2004).

The high health status in the genetic nucleus (GN) and multiplication farms at the top of the swine breeding pyramid is a barrier to genetic selection of disease resilient pigs that perform well at the lower health status found at the commercial level. To allow healthy genetic improvement, biosecurity has to be maintained at a high level to prevent introduction and any establishment of swine diseases. In chapter 2, we briefly mentioned the Cross-bred Breeding Value

(CBV) approach adopted by industry with the aim of incorporating reproductive data from commercial sows bred with semen of pureline GN sires and recording progeny performance in the commercial farms for incorporation into the GN database. This method is indirect and requires participation of commercial producers, and there are no practical tools that can directly select disease resilience pigs under such high health conditions. By using the natural disease challenge model (NDCM) (described in Chapter 2), Yorkshire-Landrace crossbred barrows from high health multiplication farms were challenged by an intense multi-pathogen challenge to simulate commercial conditions. Such a disease exposure enables animals to express their resilient genotype. Blood samples collected before challenge were sent for CBC test. Performance traits such as growth rate and treatment rate were investigated retrospectively in the resilience study.

We hypothesize that resilient and susceptible pigs generate different hematological profiles even before the occurrence of disease challenge. Our objective here was first, to reduce the dimensionality of the hematological dataset by using principal component analysis (PCA) and secondly, use these principal components (PCs) to develop a prediction algorithm that can be used in classifying pigs into resilient and susceptible groups before the occurrence of disease challenge. In this study, we also tested the impact of several types of resilient/susceptible classification (using growth rate and treatment rate of individual pig) in the outcome of these predictions.

4.2 Materials and methods

Experimental procedures were approved by the University of Alberta Animal Care and Use Committee for Livestock (AUP00002227). Pigs were handled in accordance with the guidelines described by the Canadian Council on Animal Care (CCAC, 2009). The animal study was conducted at the Centre for Research in Animal Science Deschambault (CRSAD) quarantine and

acclimatization unit in Deschambault and Centre de developpement du porc du Quebec inc. (CDPQ) test station (Quebec City, QC, Canada).

4.2.1 Animals and natural challenge model

A total of eight hundred and ninety-three (n=893) high health nursery crossbred barrows of Yorkshire and Landrace lines from multiplier farms were enrolled in this study through PigGen Canada members. The multiplier herds had been followed by a veterinarian for at least six months in order to confirm the absence or effective control of the diseases as mentioned in Chapter 2. These high health multiplier pigs were then introduced into the test station in batches of 60 or 75 pigs every three weeks. In total, fourteen batches of pigs were contributed by different PigGen members and transported from their multiplier herds to the test station. The first seven batches (batch 1 – 7) were labelled as cycle 1 and the second seven batches (batch 8 – 14) were labelled as cycle 2. These pigs were weaned at multiplier farms at approximately 21 days of age and transported to Deschambault research farm of Centre de développement du porc du Québec inc. (CDPQ) in Quebec, Canada where the natural disease challenge model was established. These barrows were introduced in batches and exposed naturally to multiple diseases simultaneously in the test station. Disease challenge was established using seeder pigs to simulate high disease pressure typical of a commercial situation. This natural disease challenge model and differences of pig flow in cycle 1 and 2 have been described in detail in Chapter 2.

4.2.2 Biological sample collection and complete blood count (CBC) analysis

Peripheral blood was collected at approximately 26-days of age via jugular venipuncture. The timing of the blood sampling was 5 days post-entry into the research facilities (cycle 1 in the

nursery unit of test station; cycle 2 in the CRSAD quarantine unit). This was also 2 weeks pre-exposure to disease challenge in the test station. Blood was drawn into EDTA tubes for complete blood count (CBC) analysis. These samples were then packaged and sent by overnight courier to University of Alberta. Out of 42 shipments, 87.8% (36) and 12.2% (5) arrived within 24 and 48 hours respectively. A standard CBC analysis was performed on the blood using the Siemens Advia 2120 (Siemens Healthcare Diagnostics, Deerfield, IL, USA). CBC traits included red blood cells (RBC), haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), red blood cell distribution width (RDW), and numbers of platelets, white blood cells (WBC), lymphocytes, monocytes, neutrophils, eosinophils and basophils (Table 4.1). Compared to chapter 3, absolute number and percentage of large unstained cells (LUC) were included in this analysis. In the end, profiling of 29 CBC traits was performed on whole blood samples collected from individual pigs before disease challenge.

4.2.3 Growth rate and treatment rate

Body weights were measured at 3-week intervals from entry to market; treatment details were recorded for individual pigs. Animals were classified into resilient and susceptible groups using growth rate and treatment rate per 100 days. Growth rate was generated using linear regression based on the body weight and age of pigs recorded in the test station at every three-week interval. Treatment rate per 100 days for an individual pig was calculated by the addition of number of injectable antibiotic administered (not including feed and water medication used on a batch basis), divided by the number of days that pig stayed in the research facility (live animal – from entry to slaughter/market; dead animal – from entry to death or euthanasia), multiplied by

100 days. Treatment of illness caused by physical trauma or any other causes that were not related to infectious diseases (traumatic injuries, death during blood collection etc.) were excluded in the calculation of treatment rate.

In the end, 105 animals were excluded. This included 23 animals that died or were euthanized due to non-infectious causes, 28 pigs in batch 11 that arrived late at the research facilities and missed blood sample 1 collection and the remaining 54 animals from various batches that were without CBC data which was most likely due to clotted blood in the EDTA tube upon arrival at the lab caused by insufficient mixing immediately after collection by venipuncture.

4.2.4 Disease resilience classification

Based on the growth rate and treatment rate generated above, four different methods of resilience/susceptible classification in the training population were utilized:

Method A) Means of growth rate and treatment rate were determined for all the animals within the two cycles. A resilient animal was classified as one with growth rate above the mean value and treatment rate below the mean value of all the animals. In other words, resilient animals were defined as an animal with higher than average growth rate and less than average treatment rate. The remaining animals were classified as susceptible. Susceptible animals included all the animals that died of infectious diseases. This classification method resulted in 29.3% (231 pigs) resilient and 70.7% (557 pigs) susceptible animals.

Method B) Means of growth rate and treatment rate were determined per batch. Within each batch, resilient (RES) animals were classified as those with growth rate above the batch's mean value and treatment rate below the batch's mean value. This classification method resulted in 44.2% (348 pigs) resilient and 55.8% (440 pigs) susceptible animals.

Method C) Instead of using mean values of growth rate and treatment within cycles (method A) or mean values within batch (method B), this method used growth rate and treatment rate to form an index value for each animal. Both growth and treatment rates were given equal weight in the equation, and the index was standardized and adjusted by batch.

Batch adjusted index = 0.5 (growth rate) – 0.5 (treatment rate)

Based on this index, animals in the upper 50% were classified as resilient, and lower 50% were classified as susceptible animals. This method therefore resulted in an equal number of resilient and susceptible animals, 50% (394 pigs) animals in each group.

Method D) Using the index method described in method C, however, instead of using the index to classify animals, it was treated as a continuous trait.

4.2.5 Statistical analysis

For method A, B and C, a linear prediction model based on the 29 CBC traits trained using principal component analysis (PCA) (R/Princomp) and stepwise feature selection (R/glmStepAIC) identified a combination of principal components with lowest Akaike Information Criterion (AIC) to classify these pigs into groups of RES and SUS. Prediction accuracy was assessed in 2-fold cross validation for the classification of pigs into resilient and susceptible groups using R/caret. Prediction accuracy based on actual outcomes was then compared with that obtained using a random classifier using Binomial test (R/binom.test).

For method D, a linear prediction model based on the 29 CBC traits trained using principal component analysis (PCA) (R/Princomp) and stepwise feature selection (R/lmStepAIC) identified a combination of principal components with lowest Akaike Information Criterion (AIC). A predicted index value was then assigned to every pig in the test population. This prediction was

then evaluated by Pearson's correlation test between actual index and predicted index value and the root mean squared error (RMSE).

Based on each prediction results in method A, B and C, the practical use in the field was examined. This was done by comparing the performance traits (number of dead animals, growth rate, treatment rate, slaughter weight and days to market) between predicted resilient and predicted susceptible groups in each method.

4.3 Results

PCA results showed that first five & ten principal components (PC) explained more than 60% & 80% of variation in CBC traits respectively (Figure 4.1). The first and second PCs explained 18.1% and 14.7% of the variations in the CBC trait dataset (Figure 4.2).

For method A, stepwise feature selection identified 8 out of 29 PCs with the lowest AIC best predict the classification of the resilience groups. This prediction model yielded an average prediction accuracy of 68.6% in a 2-fold cross validation, which was significantly ($P < 0.002$) higher than the prediction accuracy of random classifier, 58.5%. Actual and predicted classification results were presented in a confusion matrix (Table 4.2).

For method B, prediction model yielded an average prediction accuracy of 56.5% in a 2-fold cross validation, which was significantly ($P < 0.001$) higher than the prediction accuracy of random classifier, 50.7%. Actual and predicted classification results were presented in a confusion matrix (Table 4.3).

For method C, the prediction model yielded an average prediction accuracy of 55.0% in a 2-fold cross validation, which was significantly higher ($P < 0.004$) than prediction accuracy of

random classifier, 50.0%. Actual and predicted classification results were presented in a confusion matrix (Table 4.4).

For method D, the prediction model yielded a non-significant (P = 0.2031) correlation coefficient of 0.042 with an RMSE of 0.8603.

Comparison between prediction accuracy obtained in each method (A, B and C) with the prediction accuracy of respective random classifiers yielded significance difference (Table 4.5). Method A yielded the highest prediction accuracy of 68.5% in the classification of resilient/susceptible animals, followed by 56.5% in method B and 55.0% in method C (Table 4.5). In method B, there were significance difference between predicted resilient and predicted susceptible in treatment rate (P = 0.05) and days to market (P < 0.05) (Table 4.5).

4.4 Discussion

Based on the definition of disease resilience, i.e. an ability of animals to maintain an optimal performance in the face of infection, growth rate and treatment rate were selected as the two most important traits that may differentiate performing and non-performing animals at the commercial level. These two traits were selected based on the commercial goal of swine producers: to produce healthy fast growing pigs in a minimal period of time and cost. Swine producers in North America practise all-in-all-out production where any delay in pig growth negatively impacts the turnover rate of a batch of pigs that eventually leads to a loss in potential profit. On the other hand, resilient pigs were expected to grow well in a low health environment, regardless of the infection status or pathogen burden within animals. In this study, growth rate was measured as the period taken for a pig from weaning to reach slaughter weight in the finishing phase. During this period, pigs with clinical signs or a general sign of unthriftiness were treated with injectable

antibiotic in the challenge model. Thus, growth rate and treatment rate were ideal traits that may accurately reflect the growth pattern and health status of an animal.

Based on these two traits, we have experimented with four (4) different methods of animal resilience/susceptible classification in the training population and its impact on the prediction results in the testing population. Batch effect was examined in all classification methods except method A. This resulted in different proportions of classified resilient and susceptible animals in each method. Although method A yielded the highest accuracy among all the methods, methods B, C and D where 788 pigs were batch was adjusted to normalize the variations between batches were considered better options. Removal of batch effect helped in reducing variations originating from seasonality differences during batch introduction into the test station, transportation stress that depended on the distance between multiplication farm and CDPQ farm, age and weight at weaning/entry into test station and genetic background of the pigs. Batch-to-batch variation in pathogen exposure may not be evident in the CBC dataset as these pigs were not exposed to pathogens before or during blood collection, yet this variation influenced the classification of disease resilience as it was based on the performance traits, growth rate and treatment rate.

Although both method C and D were batch adjusted, animals were given an index generated by an equation whereby growth rate and treatment rate were given an equal weight. A possible disadvantage of using an index method in classification was animals with high index value could either be, i) having a high growth rate and low treatment rate (which was what we wanted), ii) high growth rate alone, or iii) low treatment rate alone. In the third scenario where animals were classified as resilient with a very low treatment rate alone may not be accurate as growth rate was not considered. In the challenge model, the growth pattern of a pig can be objectively recorded and represented in growth rate, however there could be biases in treatment rate. These biases could

be introduced by human factors, which may for example depend on the workload of the day and/or technical skills of technicians. For example, a day with higher workload or failure to recognize a clinical sign may reduce the number of antibiotic injections made per day. Therefore, in method A and B where resilient animals were first sorted out to have growth rate above mean value and then treatment rate below mean value within cycle and batch respectively would comparatively be more accurate in the resilient/susceptible classification.

Although accuracies in methods A and C yielded a value of statistical significance when comparing to accuracies in random classifiers in a Binomial test, there were no significant difference in performance traits between predicted resilient and predicted susceptible groups (Table 4.5). Predicted index obtained in Method D also yielded a non-significant low correlation coefficient ($r = 0.042$, $P = 0.2031$) with a high RMSE value (RMSE = 0. 8603). This indicated a poor fit of the predicted model to the data. In method D, the generated predicted index matched poorly to the real index. Using method B, predicted resilient animals have significant lower treatment rate ($P = 0.05$) and shorter days to market ($P < 0.05$), this result looked promising in terms of its practical use in the field data (Table 4.5). Here, it was observed that different classification methods that resulted in different proportions of resilient and susceptible pigs presented different prediction outcomes.

Before developing a prediction model, CBC dataset with 29 traits was transformed into 29 sets of orthogonal variables called principal components (PCs). The first PC placed more weight on hemoglobin, hematocrit, red blood cell distribution width, mean corpuscular volume, mean corpuscular hemoglobin, platelet, hemoglobin distribution width and reticulocyte (# & %) (Figure 4.2) and explained 18.1% of variation in the CBC dataset. The second PC placed most of its weight on neutrophil-lymphocyte ratio, neutrophil (# & %), lymphocyte (%), and total white blood cells

(Figure 4.2). The dimensionality reduction of CBC data made the subsequent classifier system more effective as expected from the results of Polat and Günes (2007).

In method B, a linear prediction model with stepwise feature selection identified a combination of 8 out of 29 PCs best classify these pigs into groups of resilient and susceptible. This model has the lowest Akaike Information Criterion (AIC) and yielded a classification accuracy of 56.5% using 2-fold cross validation. AIC has been used as a measure to rank and compare competing prediction models generated using stepwise feature selection that best approximate the true biological processes happening within the CBC dataset (Symonds et al., 2011). Classification performance of the current CBC-PCA classifier were displayed by using a confusion matrix (Table 4.3). In the confusion matrix, each cell contained the number of predicted and true resilient and susceptible pigs. Sensitivity and specificity values obtained by the model were 44.9% and 62.6% respectively.

4.5 Conclusion

In this study, although the same set of CBC and performance data were used, classification methods (A – D) played a significant role in determining the outcome of prediction results. Batch-adjusted classification should be used to normalize the variations derived from batch effect in this study as infection/disease fluctuated with the changes in pathogen load within challenge model and differences in seasonality. Classification in method B with the CBC-PCA classifier looked promising in the selection of resilient and susceptible animals before disease challenge. However, such application of CBC is novel and thus requires further investigation. Approaches such as using different classification methods, addition of more study animals or using a different computational intelligence technique (other than PCA) should be explored to validate this preliminary result and

its potential use in selection of resilient and susceptible animals. In conclusion, a significant difference in prediction accuracy between the prediction model and random classifier may not guarantee a practical use in the field. Real commercial performance should be used to validate the usability of these prediction models.

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Table 4.1 Red blood cells, white blood cells, platelet and related traits calculated from or related to these three main cell types measured in complete blood count from whole blood collected before natural disease challenge

Complete blood count		
Red blood cells[1]	White blood cells[2]	Platelet[3]
RBC count, $10^6/\mu L$	WBC count, $10^3/\mu L$	Platelet, $10^3/\mu L$
Hemoglobin, g/dL	Neutrophils, $10^3/\mu L$	MPV, fL
Hematocrit, %	Lymphocytes, $10^3/\mu L$	PDW
MCV, fL	Monocytes, $10^3/\mu L$	PCT
MCH, pg	Eosinophils, $10^3/\mu L$	
MCHC, g/dL	Basophils, $10^3/\mu L$	
CHCM	N:L ratio	
RDW, %	Neutrophil, %	
HDW	Lymphocyte, %	
Reticulocyte count, $10^3/\mu L$	Monocytes, %	
Reticulocyte, %	Eosinophil, %	
	Basophil, %	
	LUC, $10^3/\mu L$	
	LUC %	

[1]RBC = red blood cells; MCV = mean corpuscular volume; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MCHC = mean corpuscular hemoglobin concentration; CHCM = Cell hemoglobin concentration mean; RDW = RBC distribution width; HDW = Hemoglobin distribution width.

[2]WBC = white blood cells; N:L ratio = Neutrophil:Lymphocyte ratio; LUC = large unstained cells

[3]MPV = mean platelet volume; PDW = platelet distribution width; PCT = plateletocrit.

Table 4.2 Confusion Matrix (method A)

		Predicted	
		RES	SUS
True	RES	12	106
	SUS	18	261

Table 4.3 Confusion Matrix (method B)

		Predicted	
		RES	SUS
True	RES	62	76
	SUS	97	162

Table 4.4 Confusion Matrix (method C)

		Predicted	
		RES	SUS
True	RES	94	99
	SUS	79	125

Table 4.5 Pigs performance between predicted RES and predicted SUS groups in Method A, B, C and D.

Methods	Method A			Method B			Method C		
Prediction accuracy[1]	68.5% (P < 0.002)			56.5% (P < 0.001)			55.0% (P < 0.004)		
Performance Traits	Predicted RES	Predicted SUS	P	Predicted RES	Predicted SUS	P	Predicted RES	Predicted SUS	P
Number of dead animals	5	43	-	22	26	-	18	30	-
Growth Rate (g/day)[2]	755.2	762.5	0.666	765.4	757.3	0.439	760.1	762.5	0.825
Treatment number per animal per 100 days[2]	1.2	1.0	0.217	0.9	1.1	0.050	1.1	1.0	0.256
Slaughter weight (kg)[2]	120.5	122.9	0.285	122.6	123.3	0.587	122.8	122.9	0.939
Days to market (days)[2]	181.2	181.8	0.616	180.7	182.6	0.027	182.0	181.3	0.449

[1]Prediction accuracy of each method and comparison to random classifier using Binomial test
[2]Performance traits do not include those from dead animals.

Figure 4.1 Plot showed the cumulative proportion of variance explained by number of principal components.

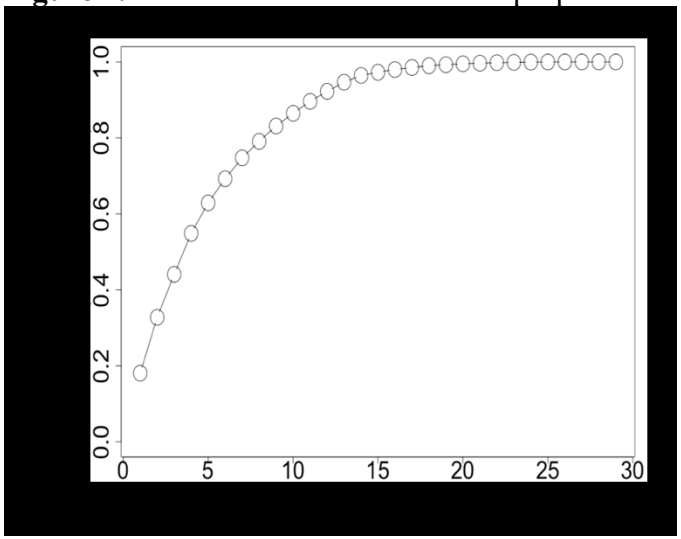
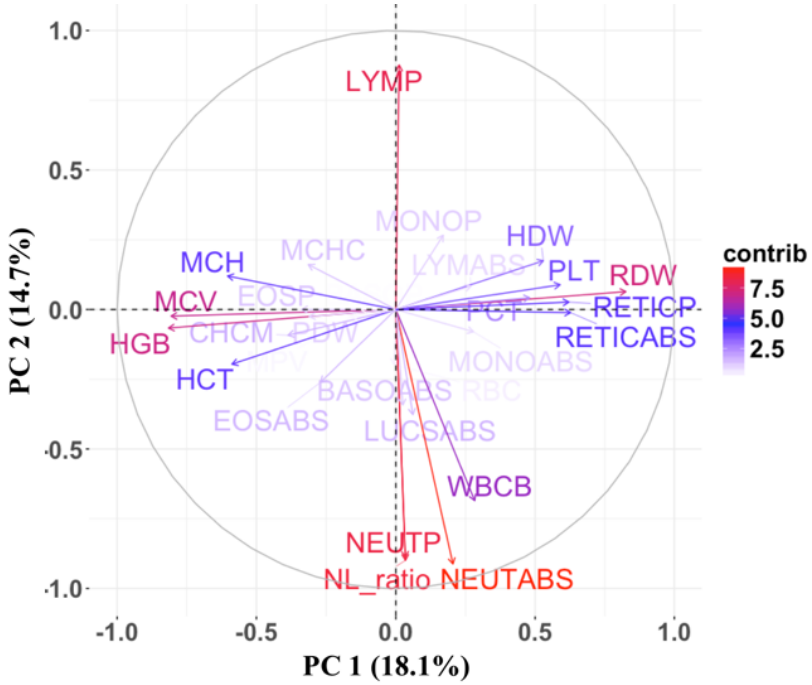


Figure 4.2 Biplot showed 32.8% cumulative proportion of variance explained by the first two principal components, PC 1 (18.1%) and PC 2 (14.7%). *Abbreviations of CBC traits as provided.



Chapter 5. General Discussion

5.1 Research summary and conclusions

The existence of considerable host genetic variance to disease in pigs has been well documented (Bishop et al., 2010). These variances, contributed in large part by the variability of host immune responses to infection, have also been demonstrated under experimental conditions (Bishop, 2010; Clapperton et al. 2009; Flori et al., 2011 and Mallard et al., 1992). Immune responsiveness is a measurement of an animal's competency in immunity. In addition, the ability of animals to respond to any infection in a way that minimizes the impact of disease has now been termed as resilience (Plastow, 2016). The advancement of genomics technology has created opportunities to explore the genetic variation of the resilience trait and will have a great impact on this costly and difficult to measure trait (Hayes and Goddard, 2010). This is also aided by the significant developments in the collection and analysis of field data, and measurement of phenotypes associated with resilience in pigs (Bishop et al., 2010). Our current resilience project combines the use of genomic technology, field data collection, immune response technology, traditional measures of productivity (e.g. growth rate and feed consumption) and natural disease challenge environments that offer immense opportunities for selection to improve disease resilience in pigs. These data, together with pedigree information and SNP genotypes, will be used to increase our understanding of the immune response and disease resilience. It will also be used in identifying the genomic regions associated with immune response, as well as the genetic relationship between immune measures on pigs in high-health environments and in health-challenged environments.

The structure of the pig production pyramid where genetic nucleus (GN) and multiplication farms are at the top of the pyramid where they are maintained in a high health status makes it impossible to select resilient pigs that are able to achieve their genetic potential in the lower health commercial level. This is evidenced by a considerable gap in the performance of breeders measured in the GN/multiplication farms and the performance of their progeny observed at the commercial level. To tackle this issue, a natural disease challenge model (NDCM) has been designed to overcome the failure in identification of resilient pigs in the GN/multiplication level. Unlike previous studies that were focused on a disease resistance approach, studies in this thesis used a disease resilience approach that was more pragmatic as it focussed on the impact of performance from disease exposure and not the host pathogen load which is relatively more difficult and expensive to measure. The NDCM to date has presented an unprecedented platform for researchers to extract these valuable phenotypes from 893 pigs in 14 batches. In chapter 2, we observed that NDCM has been successful in introducing variation in body weight gain, mortality, morbidity etc. The collection of valuable phenotypes, both performance data and biological samples were vital in characterizing the disease resilience trait in pigs.

Complete blood count (CBC), a direct measurement of immune response in an animal, has been an important extension of physical examination used by clinicians to derive a prognosis (Jones and Allison, 2007). CBC measures the concentration of three main cell types in the blood, red blood cells (RBC), white blood cells (WBC), and platelets. Five WBC differential counts by cell types (neutrophil, lymphocyte, monocyte, eosinophil and basophil) were also measured in CBC. On the other hand, cytokines are key regulators in host defense against pathogens. These proteins are potent immunomodulatory molecules that act as mediators of inflammation and the immune response. In chapter 3, we explored the CBC profiles of resilient and susceptible pigs

before and after natural challenge. We hypothesized that there are differences in CBC profiles between the two groups of pigs, and that these differences may help identify and explain the ability of resilient pigs to recover from infection or reduce the impact of disease. Chapter 3 revealed that red blood cells and platelets did not provide any insights for the resilience trait. However, resilient pigs had a higher neutrophil level before challenge and susceptible pigs experienced a persistently increasing neutrophil level after challenge. From the CBC results, we noticed that susceptible pigs experienced leukocytosis and higher neutrophil-lymphocyte ratio than resilient pigs after disease challenge. We hypothesized that this phenomenon was caused by a higher level of expression of pro-inflammatory cytokine genes in susceptible pigs. This second hypothesis was further supported by the findings of a higher level of expression of the pro-inflammatory cytokine genes, *TNF*, *IL8* and *IL1B* in susceptible pigs after challenge. Both CBC and cytokine gene expression supported the notion that susceptible pigs experienced a higher and persistent inflammatory response after natural challenge compared to resilient pigs.

A CBC profile that includes white blood cells, red blood cells and platelets has been used by researchers to develop a prediction algorithm to rank the resistance of sheep to *Haemonchus contortus* and further they were able to classify these sheep into resistant and susceptible groups with 80% and 100% accuracy respectively (Andronicos et al., 2014). Studies done in pigs have shown that persistent and low *Salmonella* shedders in fecal samples exhibited different immune response profiles as measured by peripheral cytokines and whole blood gene expression within the first 2 days of infection (Knetter et al., 2014; Kommadath et al., 2014). Furthermore, several candidate genes have been found to be associated with the shedding trait even before the beginning of a *Salmonella* challenge (Kommadath et al., 2014). The results presented in these studies support the notion that there is a practical potential for using blood samples taken before a disease

challenge in predicting a disease outcome or trait of disease resilience of an animal. The CBC test offered a direct measurement of immune response of an animal. This test is relatively inexpensive and the equipment readily available which means it could be easily implemented in the industry if it is proven to be useful. Several computational intelligence techniques have been used to develop prediction models. This is usually done by training on an initial dataset and validating or predicting on an independent dataset. These "classifier systems" have been researched extensively for medical diagnosis in human medicine and for example used in early detection of heart disease and diabetes (Polat & Günes, 2007; Nahar et al., 2013).

Here, we employed a technique of Principal Component Analysis (PCA) in order to reduce the dimensionality of the CBC dataset. PCA is a multivariate technique that has been used to extract important information where observations were described by inter-correlated quantitative dependant variables. Linear regression with stepwise feature selection was then used in the selection of a combination of principal components that best predict the class outcome. We hypothesized that resilient and susceptible pigs generate and present different hematological profiles even before the occurrence of disease challenge and that this CBC profile can be used to develop a prediction algorithm for the resilience classification of the animals. Chapter 4 revealed that with the first two cycles of animals in the NDCM such a classification method could play a significant role in predicting the outcome of the challenge. However, care is required at this point, as a significant difference in prediction accuracy obtained using CBC-PCA based prediction model and random classifier may not guarantee practical use in the field. Real commercial performance should be used to validate the usability of these prediction models. Application of CBC is novel and thus requires further investigation.

The results from this thesis have provided valuable insights on CBC characteristics for both resilient and susceptible pigs. Phenotypically, it is clear that susceptible pigs may undergo an uncontrolled inflammatory response after disease challenge. Although the link between the degree of inflammatory response and performance is not directly established, excessive inflammatory response may ultimately be the cause of a lower slaughter weight, longer time to market and higher treatment rate in susceptible animals. This thesis has also provided an insight on the potential in phenotypically predicting/sorting pigs based solely on their CBC data in an unchallenged/high-health environment. This could potentially be used as one of the tools in advancing the concept of precision livestock farming where pigs are sorted based on its disease susceptibility and subjected to different types of management (vaccination, medication, feed, stocking density etc.) to optimize the productivity of each animal. More importantly, breeders could utilize this tool to select for resilient pigs in the GN/multiplication farms. A practical tool of this sort does not exist yet, such a tool based on CBC will potentially overcome the barrier of selecting resilient animals in a high health environment.

5.2 Limitations

Overall, the research conducted provided insight into the changes of CBC profiles and innate and adaptive cytokine gene expression in resilient and susceptible pigs under the natural disease challenge model. Preliminary results also suggested that a prediction model formed using CBC parameters before challenge could potentially be applied in the field, however, further validation is required. Some challenges did exist throughout the project. In chapter 3, all the pigs selected for the analysis were from cycle 2, and classification of resilient and susceptible pigs was done within this cycle. Pigs in cycle 1 were not included due to different management (quarantine

unit and PCV2 vaccination) as compared to cycle 2 (described in chapter 2). In addition, batch effect was also not adjusted for in this study. There were seven batches of pigs in cycle 2 that were introduced at different times every 3 weeks. This batch effect was confounded by the seasonality differences during introduction, genetic background and environmental pathogen load between batches. This aspect is included in the overall design by ensuring that different sources of genetics are delivered across seasons. As there are seven suppliers this requires several cycles. Consideration of batch effect should certainly be done as the available data increases and this may further improve the accuracy of classification between resilient and susceptible pigs within the cycle.

In chapter 3 and 4, pigs were classified into resilient and susceptible groups based on the growth rate and treatment rate of the pigs. Growth rate was calculated from the time pigs were introduced into the quarantine unit post-weaning to the finisher phase in the natural challenge model. Performance records in the multiplication farms such as birth weight were not available during the time of the analyses, therefore it could not be used. Inclusion of birth weight may further improve the classification of animals as it may be correlate to and can affect the overall growth rate of pigs. In addition, we do not have the individual feed and water intake records in the nursery phase where the initial natural challenge occurs. In chapter 4, 893 pigs in cycle 1 and 2 from 14 batches were included in the study. There were some differences in the setting of natural disease challenge model between these 2 cycles that make a significant difference in the performance of the pigs as well as the levels of morbidity and mortality. In cycle 1, several batches of pigs experienced more than 50% mortality rates as well as high morbidity rates, which is not common in the commercial environment. To rectify the issue, an independent quarantine unit was set up and porcine circovirus 2 (PCV2) vaccine was applied to all pigs in cycle 2 which greatly reduced

the mortality and morbidity rates. Owing to this, some measures were taken to consider this cycle effect by using cycle 1 as training population and cycle 2 as the testing population, instead of random sampling or using batch as sampling method in our prediction model analysis. However, the best measure could have been to exclude animals in cycle 1 and to include animals from cycle 2 and the subsequent cycles that will be available in the future which had more consistent performance.

5.3 Future research

Future research can focus on finding the estimation of the heritability and genetic correlation of these CBC traits. Ideally, these traits have to be heritable under high health conditions and correlate to performance traits in commercial production to be used as predictors. In addition, by using genomic technology, SNPs or genomic regions associated with immune response and disease resilience can then be used as genetic markers that can predict resilience of the animal towards a wide range of infectious diseases. The joint effects of these SNP markers across the entire genome can then be used to calculate the genomic estimated breeding values (GEBV), i.e. genomic based breeding decision (Hayes and Goddard, 2009).

In our results, we found that certain CBC traits such as hemoglobin, absolute number of lymphocytes, monocytes and reticulocytes differs significantly between these two groups of pig. These findings presented an opportunity to further examine subpopulations of lymphocytes using different samples and/or methods. For example, types of lymphocyte subpopulations can be further dissected using flow cytometry and biological samples such as the peripheral blood mononuclear cell (PBMC). Previous research has associated CD16+, CD2+/CD16+ and CD8+ lymphocytes with the ability of pigs to grow efficiently with better carcass weight in a low health commercial

environment (Galina-Pantoja et al., 2006). On the other hand, cytokines play an integral role in the animal immune response and are involved in diverse functions, such as inflammatory response, innate and adaptive immunity etc. Therefore, various pathological conditions will be accompanied by changes in cytokine levels. These PBMC samples present an opportunity to explore the differences in the measurement of *in-vitro* production of cytokines from PBMC (between resilient and susceptible pigs) for example after stimulation with different mitogens.

Other biological samples that were collected at the same time were plasma samples, extracted whole blood samples, and whole blood in tempus tubes. These plasma samples were extracted from whole blood in 3 bleedings (B1, B3 and B4), in addition plasma samples from six age and pen-matched pigs were collected over a wider timeframe (after B1) from clinically healthy and sick animals. Differences in the level of cytokines (e.g. IL-1B, IL-8, IL-6 etc.) and acute phase proteins (haptoglobin, alpha-1 acid glycoprotein (AGP), C-reactive protein (CRP) etc.) between resilient and susceptible pigs can be tested using these plasma samples for further characterization of immune response of the two groups of pig.

Whole blood samples in tempus tubes collected during this project provide an opportunity to explore the blood transcriptome using next-generation sequencing techniques, such as RNAseq. Compared to the cytokine gene expression experiment conducted in chapter 3 (using qPCR), RNAseq provides a bigger picture by revealing the presence and quantity of RNA in the whole blood at any one time. By analyzing the continuously changing blood transcriptome, gene expression of immune-related genes (other than cytokines done in chapter 3), and growth-related genes etc. that are associated with the resilience trait can be investigated. Previous work has suggested that whole blood transcriptome has the potential to identify variation in immune response even without stimulation of the immune system (Kommadath et al., 2014). Predictor

genes that explained these variations can then be identified and incorporated in a prediction algorithm, trained, tested and validated using field data, similar to work done in chapter 4. On the other hand, RNAseq measures different populations of RNA, which include microRNA (miRNAs). MiRNAs are a novel class of non-protein-coding, endogenous small molecule RNAs which affect mRNA stability and translation into proteins (Lai, 2002). MiRNAs have been discussed as potential biomarkers in cancer and diseases such as viral infections, nervous system and cardiovascular disorder and diabetes (Wang et al., 2016). Similar to RNA, variations in miRNA profiles between resilient and susceptible pigs can be investigated and there is possibility of finding miRNAs as biomarkers for the identification of resilient animals.

Further improvement can be made in the classification of resilient and susceptible animals, these include the incorporation of daily feed consumption and water intake data available in the project. Feed consumption data of individual pigs can be used to calculate feed conversion ratio (FCR) of each pig. This would potentially complement the current classification method of using only growth rate and treatment rate, FCR generated for each individual will provide valuable information on the efficiency of conversion from feed to live weight. On the other hand, both FCR and water intake data will complement the treatment rate in defining the health status of an animal, as sick animals will experience reduction in feed and water consumption. The measurement of pathogen burden for each animal can also be another possible way to increase the accuracy of the classification. Although this measurement can be very costly, a pilot study can be conducted for a small group of animals on a specific swine pathogen to assess the practicality of pathogen burden record in the classification of animals. Also, as mentioned above, birth weight and any other variables that may be correlate to, and can affect, the overall growth rate of pigs can be accounted for to make classification of resilient and susceptible animals more accurate.

There is an on-going pilot study conducted within the challenge model that utilizes infrared thermography to measure the body temperatures of pigs in the nursery pen. Initial results revealed that a higher amount of heat emitted from the surface of the body of an immune-challenged pig as compared to a healthy pig (Fortin et al pers. comm.). The changes in body temperature were detected by infrared thermography 1.5 days before the appearance of clinical signs and temperature was correlated with disease progression in the pigs. Preventive medication can be administered earlier on these sick pigs per pen basis instead of mass medication in the entire age/phase or herd basis. This will lead to a lower morbidity and a more effective usage of medication, especially antibiotics. As mentioned earlier, the current measurement of individual feed and water intake was confined only to grower-finisher phase, this recording system can further be extended to nursery phase where the initial natural challenge occurs.

For chapter 4, the preliminary prediction results can be validated by the inclusion of more study animals (cycles 3 and the subsequent cycles), this will add more pigs into the study population. Here, we used PCA, a machine learning method in finding a linear combination of principal components that best characterized the two different classes of pigs. Alternative approaches are also possible, for example linear discriminant analysis (LDA) which is closely related to PCA where both are dimensionality reduction techniques, can be used in the same dataset. Operated using different mechanisms, PCA finds the direction that maximizes the variance in a dataset, whilst LDA computes the directions that represent the axes which maximize the separation between classes (Martinez et al., 2001). It is also not uncommon to use both techniques one after another in an analysis (Martinez et al., 2001). In another perspective, different weighting for growth rate and treatment rate can be used in method C to generate the index for each animal. With the existing biological understanding in CBC data, possible collaborations with machine learning

experts (for example from human medicine, computer science, or engineering) can be forged to further improve the prediction model.

The swine industry has long relied on vaccination, medication, management, nutrition and to a lesser extent, genetic selection programs in the improvement and prevention of many swine diseases. Recently, by using the latest gene editing technology (CRISPR-Cas9), it was possible to generate pigs with a defective CD163, the receptor for PRRSV entry into cells, so that these pigs are resistant to PRRSV (Whitworth et al., 2016). Complementing all other approaches, the finding of CD163 in PRRS resistance is a big leap forward in improving the resilience trait in pigs, although evaluation of additional pigs and viral isolates is warranted.

5.4 Conclusion

In a research perspective, striving towards producing a resilient animal, not to any one disease, but the improvement of overall disease resilience is an ambitious goal. Although there are various routes towards achieving this goal, what is lacking in the past was a platform, the natural disease challenge model, that could make all these possible. Economically, producers would be able to improve the feed conversion efficiency and incur a lower feed cost in their operations, raising a resilient animal is also the answer to providing a cheaper source of protein to an increasing world population in an environmentally sustainable manner. Both ways help in tackling food security issue that the world faces. Equally important, welfare of resilient pigs can be improved by the reduction of endemic and epidemic swine diseases, which could potentially make medication such as the usage of antibiotics irrelevant. This eventually leads to a more responsible use of antibiotics to address the global challenge of antibiotic resistance. Ultimately, technological

advancement in animal research will be the key driver in the transformation towards an improved swine health.

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